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Quadriwave lateral shearing interferometry for quantitative phase microscopy: coupling phase imaging and fluorescence imaging

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ABSTRACT

Phase imaging with a high-resolution wavefront sensor is a useful setup for biological imaging. Our setup is based on a quadriwave lateral shearing interferometer mounted on a commercial non-modified transmission white-light microscope. That allows us to make simultaneous measurement in both quantitative transmission phase and fluorescence imaging. We propose here to study co-localization between phase and fluorescence on african green monkey kidney COS7 cells. Phase permits an enhanced visualization of the whole cell and intracellular components while the fluorescence allows a complete identification of each component. Post treatments on phase-shift images are proposed and become very interesting for enhanced visualization of small details such as vesicles or mitochondrias.

Keywords: Wavefront sensor, Quantitative phase measurements, Fluorescence co-localization

1. INTRODUCTION

Functional imaging using fluorescence is a very powerful technique for biological studies. Indeed, it is possible to attach a fluorophore to a given protein : by exciting the fluorophore with a precise range of wavelengths, it emits red-shifted photons compare to the excitation. The detection of light at those wavelengths indicates the presence of a fluorophore and so the presence of the protein.
This specificity is also a limitation to fluorescence. Indeed, it is very important to know the living state of the sample before a measurement and, depending on the protein studied by fluorescence, it may be impossible to determine the vigor of the sample. To have a complete information about the sample, this technique needs to be used combined with white-light imaging; usually phase contrast techniques.
For those reasons, differential interference contrast (DIC)[1] and Zernike contrast[2] are commonly used combined with fluorescence. Those techniques present the great advantage to directly contrast the sample, by using the phase-shift introduced locally by the sample. However they only contrast the sample, and the sensibility is not tunable : it is extremely difficult to make quantitative phase measurements[3–5] .
Another approach is to use digital holography.[6] This technique measures quantitatively the phase-shift and the amplitude modulation introduced by the sample. But digital holography presents major disadvantages over DIC or Zernike contrast. In fact, digital holography is based on a Mach-Zender interferometer and requires a coherent illumination and the presence of a reference arm. The microscope needs to be entirely modified in order to implement this technique. Digital holography is also incompatible with components which alter light polarization, such as plastic elements.

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We propose here to use a wavefront sensor based on quadriwave lateral shearing interferometry (QWLSI) to measure quantitatively the phase-shift introduced by a microscopic living sample[7] . This technique presents the great advantage of being compatible with any conventional microscope as soon as it provides a video port, without any change in its optical components including the white-light source. Moreover, QWLSI can be used with plastic components such as a culture chamber for time lapse imaging.
In this paper we present the use of QWLSI for composite imaging of quantitative phase and fluorescence imaging on adherent fibroblast-like COS7 cells.

2. EXPERIMENTAL SETUP

Figure 1 schematizes our configuration.

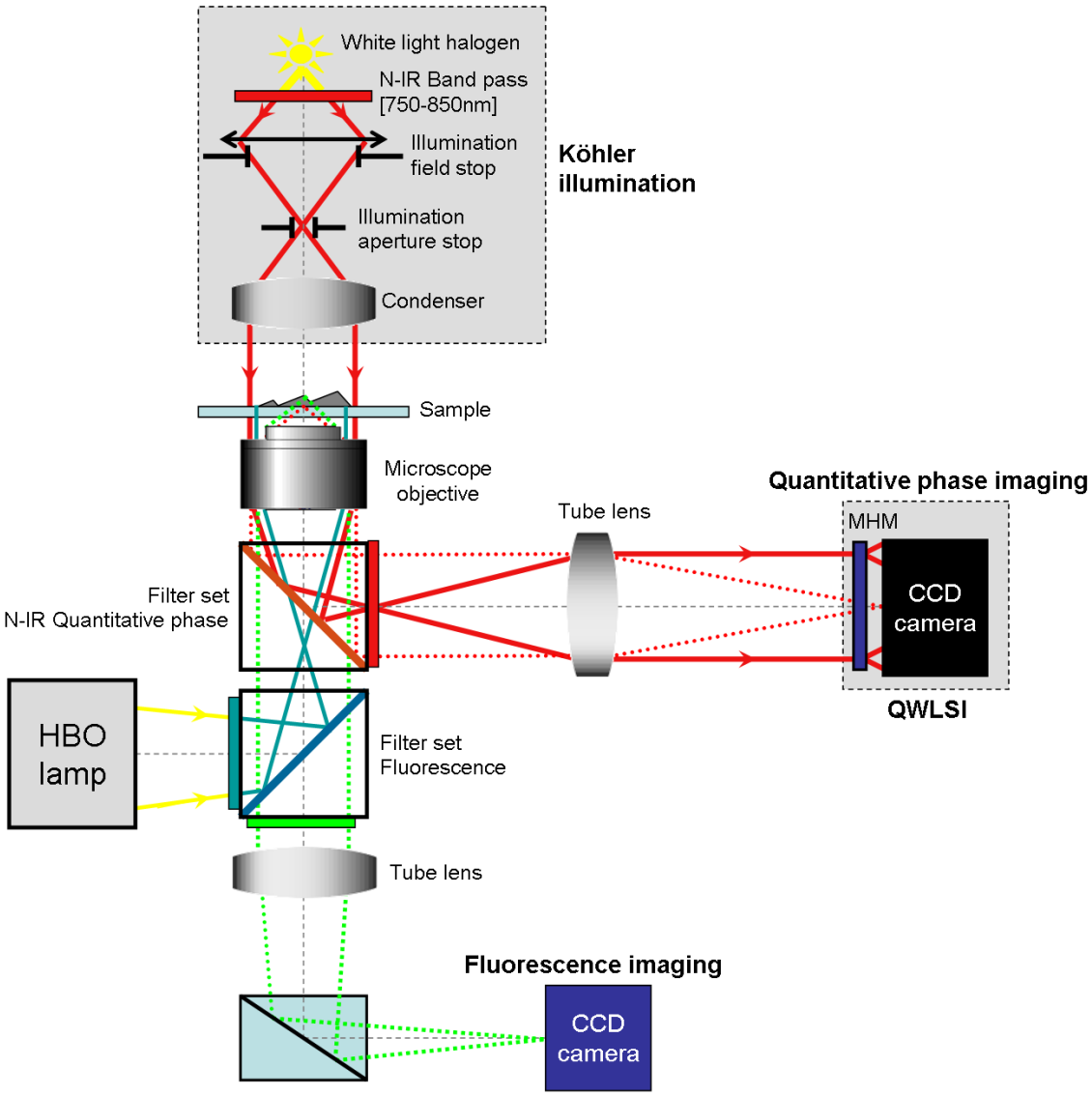

Figure 1. Scheme of the experimental setup.

In this work, we use a commercial microscope (TiU, Nikon, Japan) with an expanded space structure enabling addition of a back port combined with a second fluorescence filter turret. Such a use of an optional back port in combination with the side port allows image acquisition for two wavelengths with two cameras.
Our configuration allows simultaneous quantitative transmission phase imaging and fluorescence imaging. A band-pass filter $750nm$ to $850nm$ is added before the sample on the transmission illumination path in order to illuminate the sample in wide field with near infrared (N-IR) light. Using this spectral band has two main advantages : this is a quasi-non absorbent spectral domain for biological samples and the great majority of fluorophores are passive (no absorption and no emission) at those wavelengths, without any risk of cross talk between the transmission phase image and the fluorescence image.
The first filter holder carries a wavelength high-pass dichroic filter, reflecting light from $740nm$ to around $1\mu m$ and allowing light transmission from $350nm$ to $740nm$. This filter is dedicated to the phase imaging part : it deflects all the transmitted light without blocking the rest of visible and near uv spectrum. The second filter holder carries different types of usual filter sets, Hoechst, GFP (green fluorescent protein), RFP (red fluorescent protein) and Alexa647, in order to allow multi-marker fluorescence imaging.

A commercial quadriwave lateral shearing interferometer (SID4Bio, Phasics SA, Palaiseau, France) is used here for phase imaging. It is an easy-to-integrate and compact solution (dimension of a simple camera). The SID4Bio gives 300×400 phase and intensity measurement points with a lateral pitch of $p = 29.6\mu m$ in the image plane. The QWLSI is plugged on the microscope back exit port and measures the quantitative phase shift in the N-IR spectral band. A cooled intensity camera (Kappa DX2, Kappa opto-electronics GmbH, Gleichen, Germany) is used to visualize the fluorescence coming from the sample. The camera is plugged to one of the lateral microscope exit port. The two cameras are trigged to measure the fluorescence and the phase signal at the same moment.

As we measure quantitatively the phase-shift, a raw phase image is obtained by this method. This image can be post-processed with the help of different types of filters in order to enhance the phase-shift range of given details. For example, gradient filters can be applied to obtain a simulated DIC image along any desired direction. High-pass filtering on the quantitative phase measurement is also an interesting filter as it removes low-frequency information (global shape of the cell, or the nucleus for examples) and reveals small details (organelles, membrane ruffles for examples). In opposition to Zernike contrast, high-pass filtering is tunable and does not present any decrease in the lateral resolution.

3. CALIBRATION TO MAKE COMPOSITE IMAGES

In order to combine phase (RAW or filtered) and fluorescence images, a calibration of the camera positions is needed. Indeed, there are a residual tilt, a lateral misplacement and a differential magnification between the two detectors. Moreover, the position of the back tube lens needs to be precisely tuned in order to have a perfect imaging simultaneously on the QWLSI and on the fluorescence camera.

To solve this problem, a target is first placed in the microscope object plane and imaged with a small magnification ($10\times$). The N-IR filter placed in front of the halogen light is removed in order to illuminate the sample also in the fluorescence excitation band. The tube lens is then precisely positioned to image the same object plane on the two detectors (as shown in the figure $2(a, b)$). An algorithm is then applied to extract the exact position, angle and homothety of the phase image compared to the fluorescence image as shown in figure $2(c)$.

This kind of calibration is then applied to all measurements for phase and fluorescence imaging. The parameters are not dependent on the objective used if it is well corrected for chromatic aberrations, which is the case in our setup ($\times 100$, $NA = 1.3$ plan apochromat objective, Nikon, Japan). In the case of a chromatic objective, this calibration needs to be done for each fluorescence filter used.

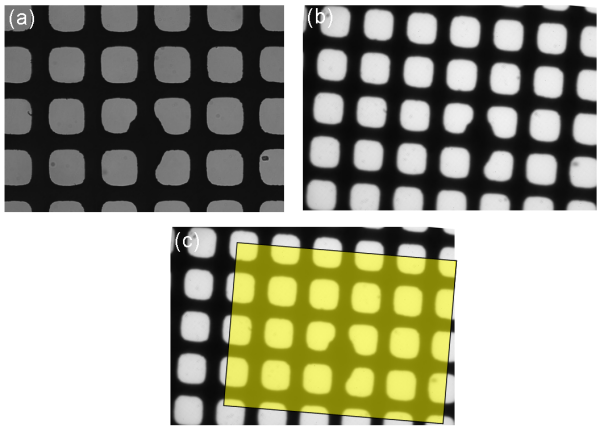

Figure 2. (a) Image of an electronic microscopy grid at ×10 on the fluorescence camera. (b) Image on the QWLSI in case of an overdone tilt of the sensor. (c) Image on the QWLSI after the application of the calibration algorithm, the yellow rectangle shows the calculated position of the fluorescence image in the field of the QWLSI image.

4. PHASE AND FLUORESCENCE COMPOSITE IMAGING

In this part, we use the setup described in the part 2 to make composite image of phase and fluorescence.

COS7 cells[8] are african green monkey kidney cells, adherent on their substrate. The geometry of such a cell is variable, with important shape modifications (ruffles, lamellipodium growth). Studying COS7 cells is interesting for studying membrane dynamics and intracellular movements.[9] We propose here to study COS7 with a ×100, $NA = 1.3$ immersion objective in order to visualize a single cell (field of view of $118 \times 89 \mu m^2$) with a good lateral resolution. The figure 3(a) shows a RAW quantitative phase image of COS7 cells imaged on the QWLSI : the optical thickness of the sample is visualized here. A post processing of this image can be done in order to remove the low frequency image information : this removes the slowly variable phase information and allows an enhancement of the small structures such as the mitochondrias or vesicles (Figure 3(b)).

Cells are transiently transfected and express different fusion proteins : a protein specific of mitochondrias fused with GFP and a protein specific of Golgi apparatus fused with RFP (CellLight, Invitrogen). Double strained DNA is tagged with Hoechst 33342 (Blue, Invitrogen). GFP image (Figure 3(c)) is recorded on the intensity camera in parallel to phase image as the mitochondria movements are faster than the DNA reorganization. Hoechst (Figure 3(d)) and RFP images (Figure 3(e)) are acquired sequentially in less than ten seconds, the Golgi apparatus is recorded first as their movements are quicker. Calibration described on the part 3 is applied to the high-pass filtered phase image and a composite image is created from the three fluorescence color plus the phase image (Figure 3(f)).

It is interesting to note that each fluorescent cell component is clearly visible on the phase image. The nucleus roughness visible in the filtered phase image is directly linked to inhomogeneity in the DNA density (visible on the fluorescence image). The Golgi can be seen on the phase as a smoother zone next to the nucleus. The correlation between mitochondria phase and mitochondria fluorescence is very strong : the filtered phase allows a clear determination of the mitochondria optical density and co-localization with fluorescence is particularly clear.

5. CONCLUSIONS

We demonstrate here the particular interest of measuring the phase-shift introduced by a sample. The use of QWLSI allows a quantitative measurement of this phase-shift without modifying neither the microscope nor the sample preparation. A huge number of cell components are clearly visible in phase (Golgi apparatus, DNA or

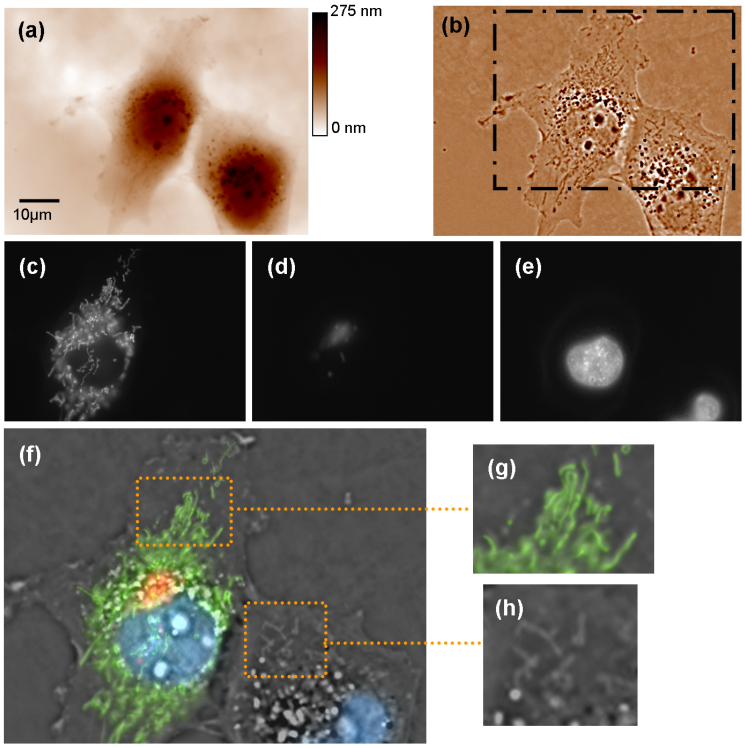

Figure 3. (a) Quantitative phase image of COS7 cells. (b) Image processing of (a) : High-pass filtering to enhance small structure ; the black dashed rectangle represents the overlap between fluorescence image and phase image. (c) Fluorescence image of GFP marked mitochondrias. (d) Fluorescence image of RFP marked Golgi apparatus. (e) Fluorescence image of Hoechst marked DNA. (f) Composite image of cropped-(b) (gray), c (green), d (orange) and e (blue). (g) Zoom on a cell zone where fluorescence of mitochondria and phase perfectly overlap. (h) Other cell wich does not express fussion proteins, where we can see mitochondrias in phase.

mitochondrias as we presented here) and fluorescence confirms that. Phase-shift gives information about those components and carries a potential interest when considering a study of density within the structure.
QWLSI is completely suitable with biologic experiments : we are not in need to modify neither the sample preparation protocol nor the experimental conditions. Fluorescence and phase co-localization with QWLSI gives many more information compared to DIC or Zernike contrast without complicating the acquisition procedure.

REFERENCES

[1] Nomarski, G., "Nouveau dispositif pour lobservation en contraste de phase differentiel," *J. Phys. Radium* (1955).

[2] Zernike, F., "Das phasenkontrastverfahren bei der mikroskopischen beobachtung," *Z. Tech. Phys.* **16**, 454–457 (1935).

[3] Hariharan, P., "Achromatic phase-shifting for white-light interferometry," *Appl. Opt.* **35**(34), 6823–6824 (1996).

[4] Xu, Y., Xu, Y. x., Hui, M., and Cai, X., "Quantitative surface topography determination by differential interference contrast microscopy," *Optics and Precision Engineering* **9**, 226229 (2001).

[5] Arnison, M. R., Larkin, K. G., Sheppard, C. J. R., Smith, N. I., and Cogswell, C. J., "Linear phase imaging using differential interference contrast microscopy," *Journal of Microscopy* **214**(1), 7 – 12 (2004).

[6] Cuche, E., Bevilacqua, F., and Depeursinge, C., "Digital holography for quantitative phase-contrast imaging," *Opt. Lett.* **24**(5), 291–293 (1999).

[7] Bon, P., Maucort, G., Wattellier, B., and Monneret, S., "Quadriwave lateral shearing interferometry for quantitative phase microscopy of living cells," *Opt. Express* **17**(15), 13080–13094 (2009).

[8] Gluzman, Y., "Sv40-transformed simian cells support the replication of early sv40 mutants," *Cell* **23**, 175–182 (1981).
[9] Sergé, A., Bertaux, N., Rigneault, H., and Marguet, D., "Dynamic multiple-target tracing to probe spatiotemporal cartography of cell membranes," *Nature Methods* **5**, 687 – 695 (2008).