MICROSCOPY

One-shot analysis

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Digital holographic microscopy's ability to reconstruct three-dimensional surface topography from a single measurement without the need for any scanning makes it extremely robust and immune to vibrations. Its applications range from bioimaging through to analysing micro-electromechanical systems devices and quality-assurance tasks.

Digital holographic microscopy (DHM) is a newcomer in the field of high-resolution microscopy. Based on the principle of holography and combined with state-of-the-art digital-image acquisition and processing technology, DHM opens the door to fast, robust, three-dimensional (3D) microscopy (Fig. 1a).

Characterized by a diffraction-limited transverse (in-plane) resolution and a subnanometre vertical (axial) resolution, DHM has many features in common with interference microscopy techniques, which also make use of a reference wave to record the phase of optical waves. But thanks to an unprecedented level of numerical processing, DHM is robust, easy to use and provides extremely fast measurement rates, making it ideal for many applications in materials and life sciences.

DHM can be configured to analyse samples in reflection (Fig. 1b) or transmission (Fig. 1c) modes. Reflection-mode DHM is used for general micro- and nano-characterization of reflective structures and surfaces, whereas transmission-mode DHM is used to study transparent or semi-transparent samples such as plastic or glass devices and biological cells.

The origins of DHM can be traced back to 1968, when Joseph Goodman at Stanford University proposed the use of a computer for the numerical reconstruction of digitally acquired holograms. However, it was only in the 1990s, when the required computer processing power became available, that this attractive idea emerged as a technology with real potential. In 1999, a group directed by Christian Depeursinge from the Ecole Polytechnique Fédérale de Lausanne (EPFL) in Switzerland, developed an innovative method for the numerical reconstruction of the phase distribution at the specimen surface, and demonstrated step-height measurement at the nanometre scale. This research led to a new imaging concept based on the digital processing of wavefronts — defined as complex mathematical entities with both amplitude and phase — rather than standard intensity images.

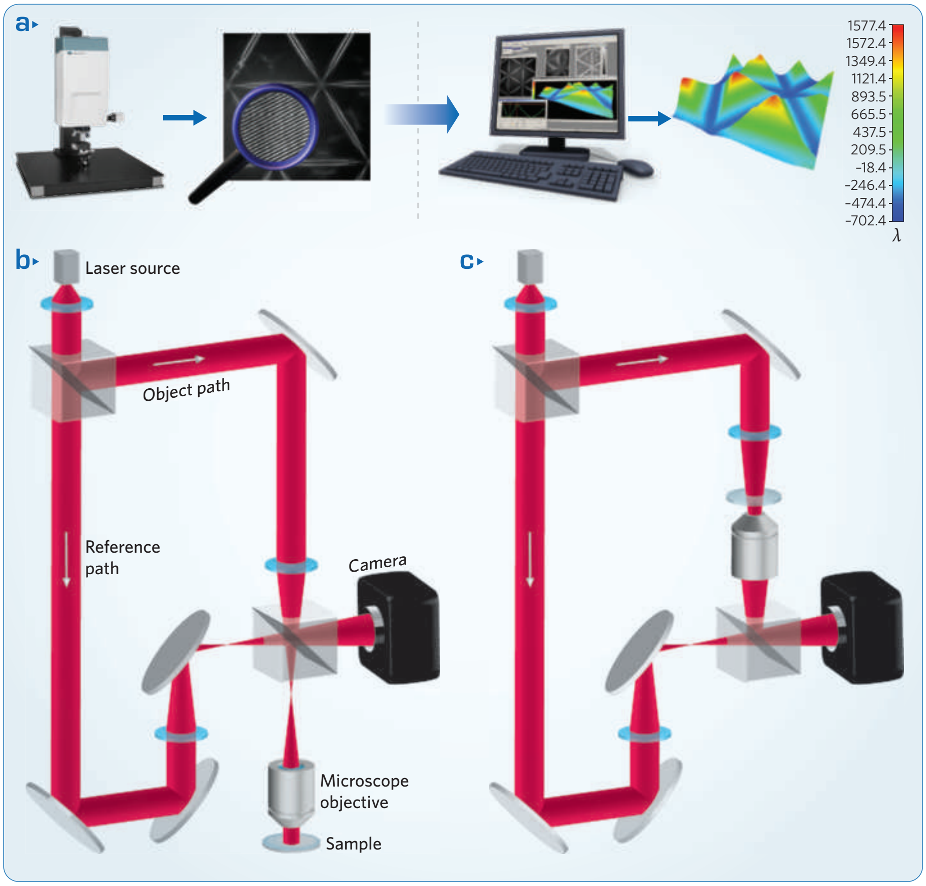

Figure 1 | Schematic of the principle of DHM. **a**, Acquisition (left): a hologram is generated by interference of a reference wave and an object wave, and is recorded on a CCD camera. Reconstruction (right): the hologram is transferred to a computer that numerically reconstructs a phase image, out of which a 3D representation is created. All of the information is recorded on a single hologram capture and the system allows real-time observations at video rate. **b**, The reflection mode of DHM, in which the surface topography is measured with subnanometre vertical resolution. **c**, The transmission mode of DHM, in which the phase shift — induced when the light passes through transparent samples — is measured.

This approach has rapidly found numerous applications, especially in microscopy. Today, DHM has acquired the status of a scientific discipline in its own right, with a large community of researchers all around the world and around 200 peer-reviewed publications every year. Commercial DHM systems have been available since 2003, with Lyncée Tec

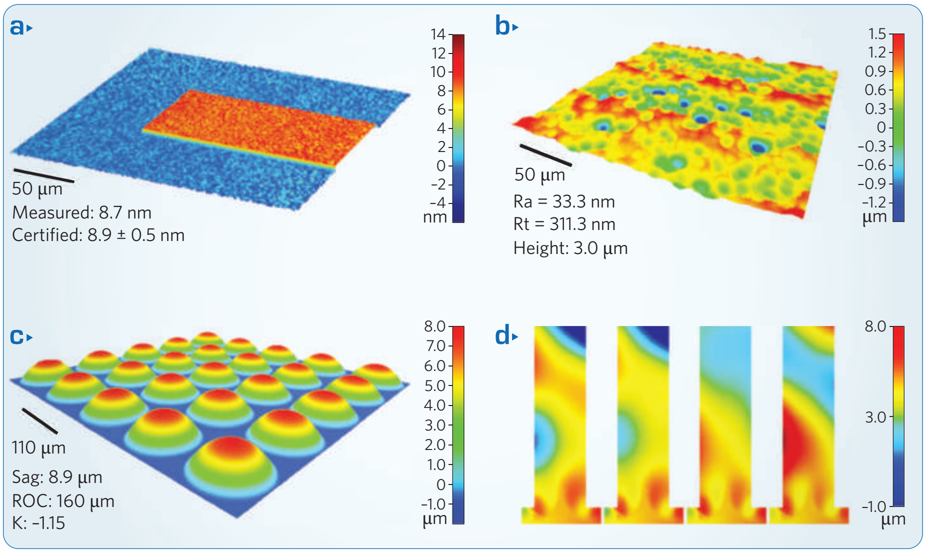

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Figure 2 | Typical applications of DHM in material sciences. **a**, A certified step-height measurement of 8.9 nm. **b**, Analysis of a rough surface. **c**, Microlens observed in transmission mode. **d**, Stroboscopic imaging mode applied to MEMS characterization, showing four positions of a cantilever at its resonant frequency (87 kHz).

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REFLECTION-MODE DHM

DHM in the reflection configuration (Fig. 1b) can be included in the same family as optical interferometric profilers, but there are considerable differences.

First, in contrast to standard phase-shifting interferometric techniques (which require several image acquisitions showing different states of a reference wave) or vertical scanning interferometry (which requires vertical scanning of the sample or objective), DHM allows 3D reconstruction of the surface topography through a single image acquisition, without any scanning. This 'one-shot' exposure feature of DHM means it is extremely robust and immune to vibrations. The very short recording time can even be reduced down to the nanosecond-scale by using a pulsed laser. A computer running optimized software allows reconstruction speeds upwards of 30 holograms per second, for holograms measuring 1,024 × 1,024 pixels. These unique temporal features of DHM offer attractive possibilities for quality-control on the production floor and for dynamical analysis of samples, in particular micro-electromechanical systems (MEMS).

Second, with DHM the calibration for vertical measurements is uniquely defined by the wavelength of the light source — a physical parameter that can be certified and stabilized with much more confidence than mechanical displacements on which standard commercial instruments using phase-shifting interferometry are based. National metrology laboratories have identified this as a key feature of DHM.

Third, digital holography microscopes are now equipped with up to three light sources of different wavelengths. By combining them, the vertical measuring range can easily surpass several micrometres, and the subnanometre resolution is preserved (Box 1).

Fourth, DHM can be used in conjunction with any conventional microscopic objective, including immersion, high numerical aperture and long working-distance optics — there is no need for specific interferometric Mireau or Michelson objectives.

Applications for reflection-mode DHM include all domains that are classically addressed by optical profilers — namely non-contact surface analysis and microdevice inspections in the semiconductor, optics, micro- and nano-

Box 1 | DHM with several wavelengths

Vertical topographic measurements are defined by phase measurements. Thus, they are limited by their wavelength, because a 2π phase change is equivalent to the wavelength in terms of distance.

In reflection-mode DHM, height measurements have a maximum limit of half the wavelength — approximately 300 nm for visible-light sources. With a smoothly varying surface, this limitation can be overcome through software procedures (such as 'phase unwrapping') that allow the retrieval of the real specimen topography even over height changes of several micrometres. However, these numerical procedures fail when analysing abrupt changes in height, such as steps or samples with steep slopes.

The first generation of DHM instruments were equipped with a single light source and so were limited in their vertical measuring range — this was a significant handicap for a large number of standard metrology applications. But DHM is now increasing its versatility by using two laser sources with different wavelengths. With such a configuration, much larger measuring ranges can be achieved by probing the specimen topography with a synthetic wavelength that results from the beating of the two sources. This enables the vertical measurement range to easily surpass several micrometres. The subnanometre resolution is preserved because the standard single-wavelength procedure is intrinsically comprised in the dual-wavelength method.

Dual-wavelength-mode DHM systems are already commercially available, but they are implemented in an alternative mode that requires two successive acquisitions while switching the two sources on and off. Such an approach decreases the acquisition rate because the 'one-shot' acquisition is lost. To preserve this feature and the remarkable speed and robustness of DHM, a new recording configuration has been developed. This process uses the well-known principle of multiplexing in holography, which enables the recording of several holograms simultaneously on the same recording media by using multiple reference waves that reach the hologram plane at different incidence angles — as if two digital holographic microscopes are nested in a single instrument. Lyncée Tec now offers digital holographic microscopes with up to three light sources and a vertical measuring range of up to 15 μm, preserving subnanometre accuracy and maintaining great speed and robustness.

technology industries. In these areas of materials science, strong competition exists between well-established technologies such as interferometric, confocal and atomic force microscopes. Even with competitive prices and growing popularity, DHM is still largely confined to niche markets where its unique ratio of speed to resolution is specifically required. Applications include surface-structure analysis of micrometre- and nanometre-scale samples such as wafers, microbeads and shell surfaces, or of metallic sheets after being passed through a rolling mill. When the use of motorized stages is combined with the 'stitching' of individual measurements, very large surfaces can be analysed very quickly.

DHM is also used in the metrology of structured oxide layers on silicon wafers, for instance in measuring the depth of craters produced by secondary-ion mass spectrometry on silicon wafers with SiO_2 thin films.

Thanks to a unique stroboscopic acquisition mode, DHM is a popular tool for static and dynamic analysis of MEMS (Fig. 2). DHM allows dynamic characterization of structures in periodic or repetitive movements at excitation frequencies up to 25 MHz, and laser pulses down to 7.5 ns can be used to image very fast-moving samples. DHM also enables analysis with subnanometre vertical sensitivity. Furthermore, investigating resonant behaviour, settling time, stability and repeatability of a wide variety of devices such as cantilevers, micropumps, accelerometers and micromirrors, as well as the measurement of, for example, thermal effects on a laser crystal, are all possible with DHM. The competitors to DHM at this level are vibrometers based on laser doppler velocimetry. These instruments are attractive for high-frequency analysis, but have the disadvantage that full-field measurements require a 2D scan of the probing beam over the specimen surface.

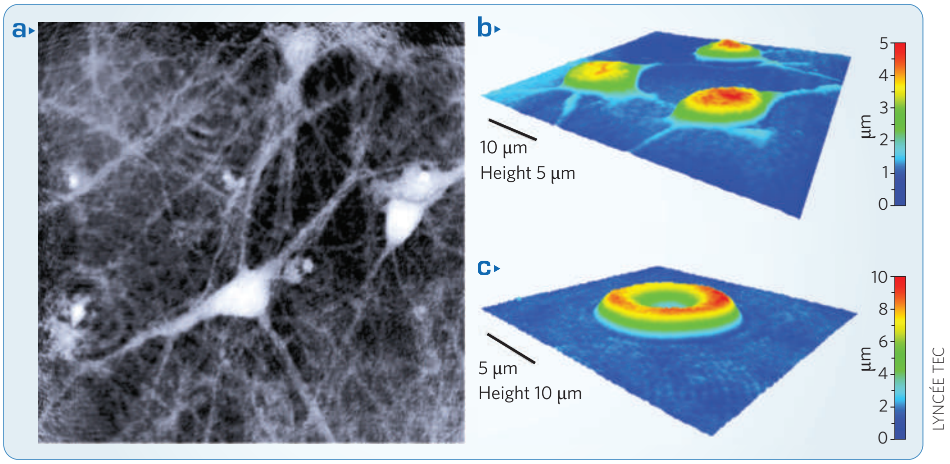

Figure 3 | Applications of DHM in cellular biology. **a**, Two-dimensional phase image of mouse cortical neurons. **b**,**c**, Three-dimensional reconstructions of non-differential PC12 cells (**b**) and a red blood cell (**c**). DHM is unique in that it allows real-time observation of cells in their physiological environment without the use of any contrast agent, such as fluorescent dyes or markers. The measured phase shifts can be interpreted, for instance, in terms of changes of cellular morphology and trans-membrane currents for neurons, and in terms of mean cellular haemoglobin, morphology, volume and cytoskeleton vibrations for red blood cells. Cells can also be tracked and volume and surface measurements can be performed.

TRANSMISSION-MODE DHM

DHM in transmission configuration (Fig. 1c) has no equivalent in the interference-microscopy market. It allows quantitative characterization of the optical behaviour of transparent samples by measuring the phase shift of the light passing through the sample, which depends on the sample thickness and refractive index. Glass or plastic devices, micro-optical components, as well as fluids and organic materials, can be analysed in this way.

One of the most important areas for transmission-mode DHM is life sciences and cellular imaging (Fig. 3). Research led by Pierre Magistretti and Pierre Marquet from EPFL has resulted in the development of techniques to interpret the very rich phase information in terms of underlying biological processes for a large variety of cells, including red blood cells, neurons and human embryonic kidney cells. Monolayers of living cells can be analysed in a strictly non-invasive way, without any contrast agents. DHM provides precise and quantitative measurements of cellular morphology (for example, cell thickness and volume) and cellular contents (such as the refractive index, protein density, dry mass and water content). Ion movement or trans-membrane currents can also be monitored with DHM. The real-time capabilities of DHM are particularly attractive for time-lapse microscopy, high-throughput screening, and diagnostics. The scheme enables the quantitative monitoring of the dynamical behaviour of cells with nanometre-scale axial sensitivity, and microsecond-scale temporal sensitivity, for example in the study of cell's response to a change in its environment (due to drug delivery, or electrical or mechanical stimulation).

Cells are complex, and a single technique is not sufficient for a full understanding of their functionalities. To meet the expectations of this emerging but highly promising market of DHM for life sciences, Lyncée Tec recently launched a multimodal DHM configuration comprising a complementary module for simultaneous video fluorescence imaging. This instrument combines the information from well-established fluorescence methods, appreciated for their specificity, with the quantitative information provided by DHM.

The next main challenge for DHM technology is to acquire the status of a 'standard method' for sample characterization at the micro- and nanoscales, which is widely recognized within the metrology and cellular-microscopy communities. Since 2005, when the technology first came to market, DHM has matured and successfully entered areas where its competitive advantages of high-speed, high-precision, robustness, ease-of-use and affordability are attractive. Modules for specific applications — for example, stroboscopic synchronization or fluorescence — have been developed to answer users' needs. As for the future, the life sciences sector offers the largest growth potential, because the ability to interpret phase images in terms of underlying biological processes is highly valued. Given that DHM components are widely used in mature technologies, particularly in photonics and computer processing, continual improvements in DHM performance should be expected in the future. ❒

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