

# DHM (Digital Holography Microscope) for imaging cells

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**Abstract.** Light interaction with a sample modifies both intensity and phase of the illuminating wave. Any available supports for image recording are only sensitive to intensity, but Denis Gabor [1] invented in 1948 a way to encode the phase as an intensity variation: the “hologram”. Digital Holographic Microscopy (DHM) [2] implements digitally this powerful hologram. Characterization of various pollen grains and of morphology changes of neurones associated with hypotonic shock demonstrates the potential of DHM for imaging cells.

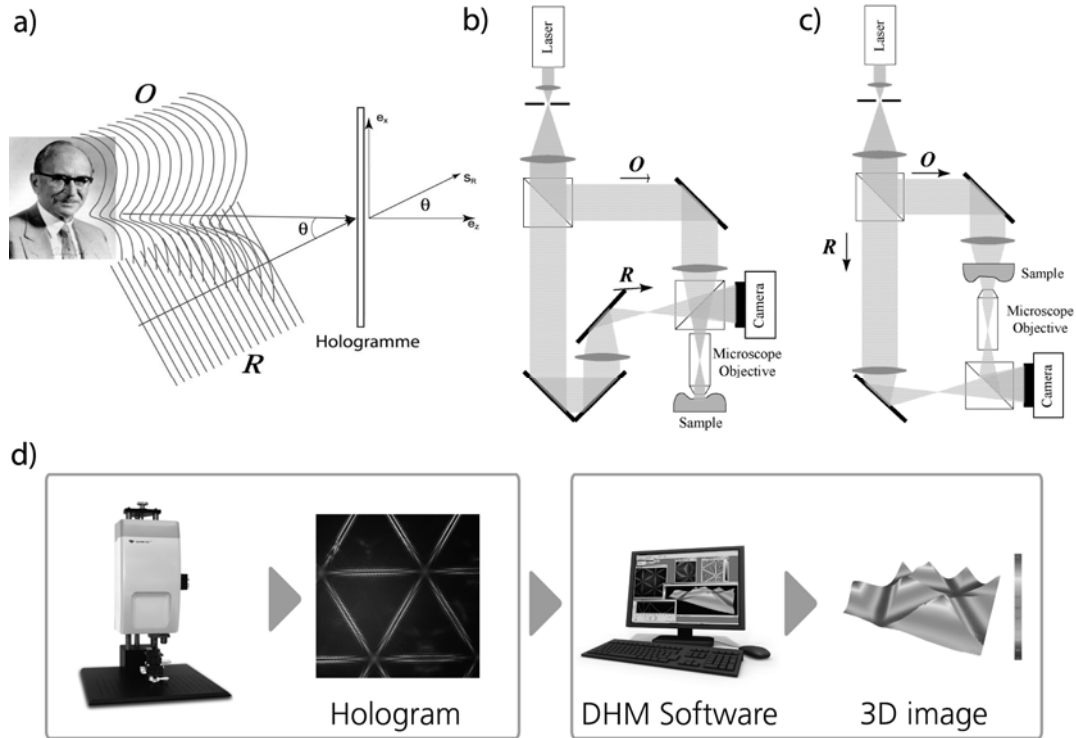
## 1. Digital Holography Microscopy (DHM)

Although any available supports for image recording are only sensitive to the intensity of the light wave, its interaction with a sample modifies both its intensity and its phase. Most of biologic microscopic specimens, called phase object, are transparent and differ only slightly from their surroundings in terms of absorbance and color: they provide a modest change in amplitude, but they alter the phase of the detected light wave.

Development of imaging techniques for non invasive visualization of unstained phase objects, has led to the development of optical phase contrast-enhancing techniques, such as phase contrast (PhC), initially proposed by Zernicke and Normarski’s differential interference contrast (DIC) microscopy, as well as of interferometric techniques. This last technology, yields quantitative measurements of the phase distribution produced by the sample, what is not the case for the two first phase contrast-enhancing techniques [1]. They are widely used in metrology, but their use has not been reported often in biology, probably due to the fact that interferometric setups involve often complex laser systems, including modulators and piezo-driven mirrors, requiring complex calibration and handling. In particular, several image acquisitions are required to retrieve quantitative measurements, what is not well suited for dynamical analysis of cell morphology changes.

Denis Gabor [2] invented in 1948 a way to encode both the phase as an intensity information in a single intensity record: the “hologram”. Digital Holographic Microscopy (DHM) in off axis configuration [3] implements digitally this powerful hologram. With the present power of computers and the developments of digital cameras, holograms, acquired in a few tens of microseconds, can be numerically interpreted to provide simultaneously: (1) the phase information, which reveals object surface with vertical resolution at the nanometer scale, and (2) intensity images, as obtained by conventional optical microscope. Both images are defined with a diffraction limited resolution in the

transverse ( $Oxy$ ) plane and are “reconstructed” from the hologram at video rate. This principle is illustrated in Fig. 1.



**Figure 1.** (a) Recording principle of off axis holograms. There are a few degrees (angle  $\theta$ ) between the reference ( $R$ ) and the object beams ( $O$ ). This enables to reconstruct the information using a single hologram acquisition. On-axis holography (i.e.  $\theta=0$ ) requires acquisition of several holograms. The portrait is the one of Dennis Gabor, Nobel Prize of physics for its discovery of Holography. (b) Optical set up for reflection DHM: the source beam is first separated into two parts: a reference beam and an object beam. The object beam illuminates the sample. The light diffracted by the sample is collected by the microscope objective and is combined with the reference beam to form a hologram on the camera. (c) Optical set up for transmission DHM: the source beam is first separated into two parts: a reference beam and an object beam. The object beam illuminates the sample. The light diffracted by the sample is collected by the microscope objective and is combined with the reference beam to form a hologram on the camera. (d) DHM principle: once the hologram captured by the microscope, it is numerically interpreted to provide intensity and phase images, allowing 3D representation of the sample.

With light irradiance smaller than  $200 \text{ mW/cm}^2$  incident on the sample, DHM provides strictly non invasive real time quantitative measurements and opens new perspectives for visualization of living cells.

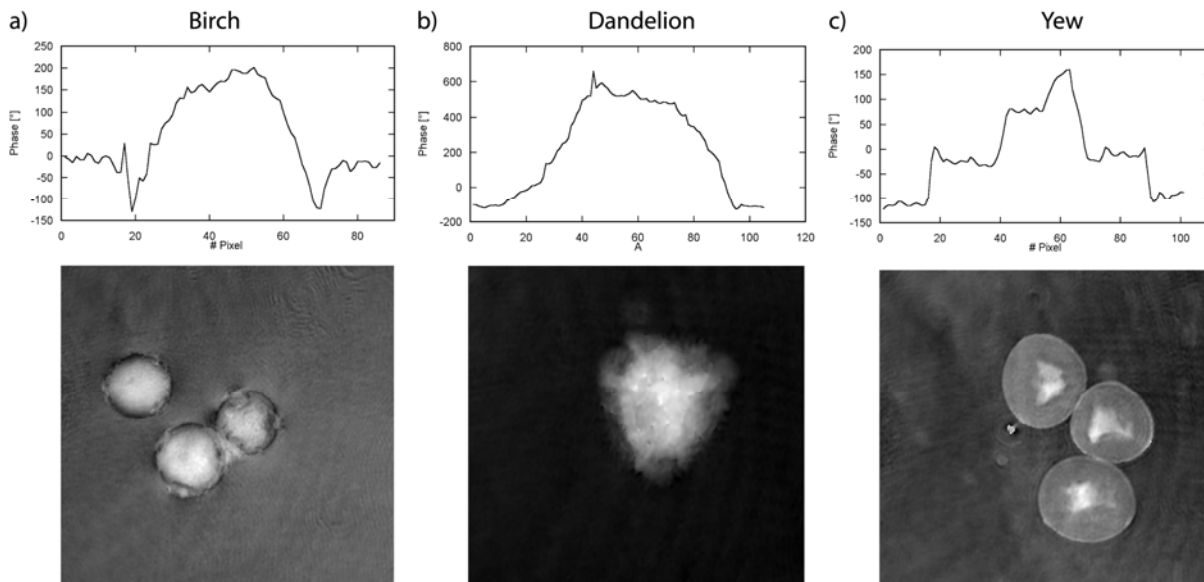
## 2. Applications in cell biology

Quantitative phase contrast mapping of the sample enable to perform living cell analysis, sorting, and recognition. By providing short acquisition time of typically 10 microseconds and fast measurement acquisition rate, limited only by the camera frequency acquisition rate, DHM enables also to dynamical investigation of natural and stimulated morphological changes associated with drug, electrical or thermal stimulation.

### 2.1. Cell characterization

Pollen particles samples, with size ranging between 20  $\mu\text{m}$  and 30  $\mu\text{m}$ , were examined. Figure 2 presents typical examples of phase images for three different pollen types: birch, dandelion, and yew. Typical profiles of three different pollen particles types are exposed. It shows clearly the uniqueness of the phase signal for each pollen type, as the refractive index is different for each cell part (membrane, core, cytoplasm) and also for each pollen type.

The negative phase difference visible on the birch pollen profile (the two depletions on both sides of the cell) can be explained by a refractive index in the membrane smaller than the extra-cellular environment (a liquid with a refractive index about 1.3). On the contrary, on the yew pollen profile, small spikes on both sides of the cell are due to a refractive index in the membrane greater than both cytoplasm and extra-cellular environment refractive indexes. On the dandelion pollen profile, neither depletion nor spikes are visible, that signifies that the refractive indexes in the membrane and in the cytoplasm are about the same value, greater than the extra-cellular environment. Their shapes, different due to specific cells architectures, and their ranges, from 200° and 250° for respectively the birch and the yew cells to 600° for the dandelion cell, can be used to achieve cell type sorting.



**Figure 2.** Different pollen grains, (a) Birch, (b) dandelion, and (c) yew measured with a DHM in transmission configuration. For each pollen, the phase measurement is shown on the bottom, and a horizontal phase profil through one grain is shown on the top of it.

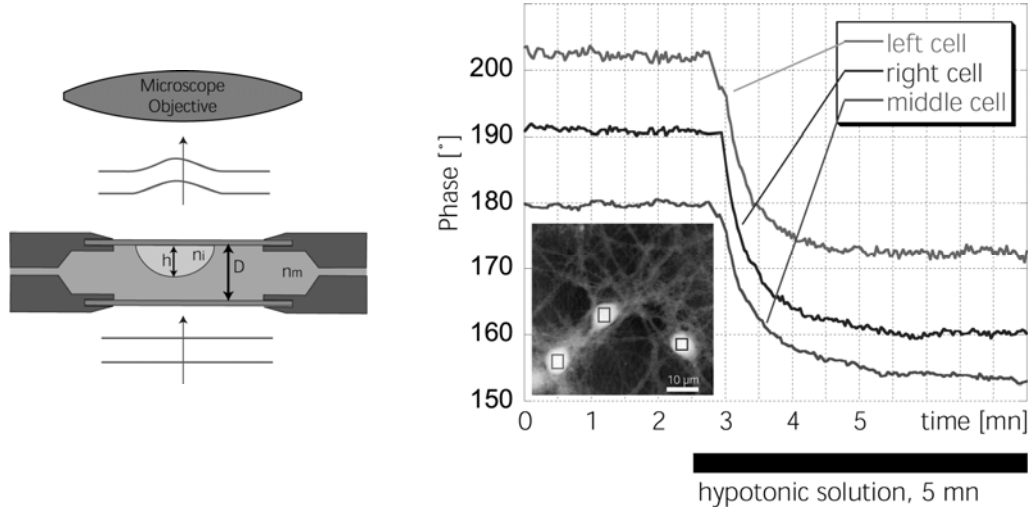
Phase measurements provide unique and quantitative signatures of the structure and of the type of pollen grains. This application illustrates that DHM provides unique and quantitative criterion for analysis and recognition of living biological specimens.

### 3. Dynamical investigation of cells

Primary cultures of mouse cortical neurons are prepared from mouse E17 embryo and plated on poly-L-ornithine coated coverslips in Neurobasal + B27 medium. Neurons are used after 2-3 weeks in culture. Coverslips are mounted in a closed perfusion chamber used to apply the different solutions to the cells (See Figure 3, left panel).

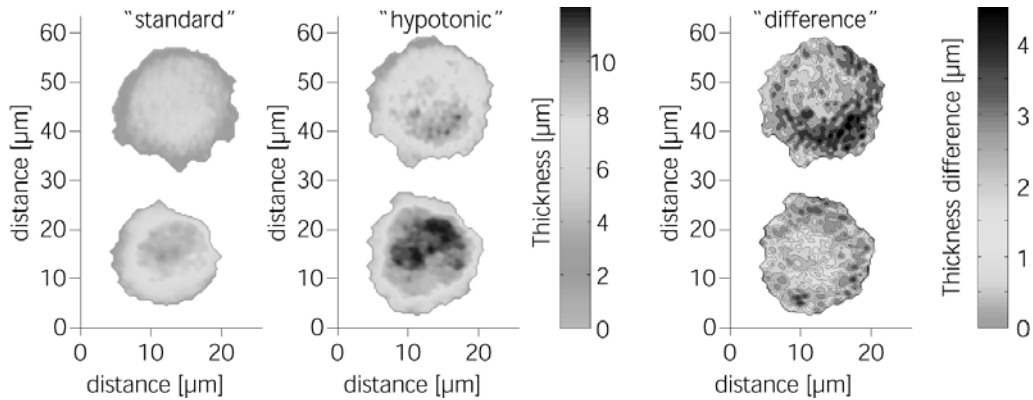
An hypotonic shock on primary cultures of mouse cortical neurons is achieved by replacing a standard perfusion solution (229 mOsm/kg H<sub>2</sub>O) by a hypotonic solution (144 mOsm/kg H<sub>2</sub>O) reducing the extracellular osmolarity by 37 %, which represents a relatively high stress for the neurons. As depicted in the right panel of Fig. 3, the hypotonic solution produces a drop in the phase

signal, which reaches a plateau after 2 minutes. Such phase decrease remains difficult to be interpreted as a cellular swelling. To investigate this a priori paradoxical phase behavior, a decoupling procedure [4] is applied to separately measure the mean integral refractive index along  $z$  and the thickness of the neuronal cell bodies.



**Figure 3.** Left panel: Perfusion chamber. The cells are attached to the cover slip. Perfusion is performed through the input and output outlet of the chamber (left and right opening). The perfusion medium and the cell are characterized optically by their thickness ( $D$  and  $h$  respectively) and their refractive index ( $n_m$  and  $n_i$  respectively). The change of phase of the wave associated with the sample is illustrated. Right panel: Real-time monitoring of the phase signal of 3 neurons observed during a hypotonic shock. Inset: quantitative phase contrast image of the monitored neurons. The phase mean values of the rectangles are plotted versus perfusion time. The black bar denotes perfusion of the hypotonic solution for 5 minutes.

The obtained morphometric images indicate clearly the expected hypotonic neuronal swelling. The hypotonic-mediated shape variations can be clearly seen in middle panel of Fig. 4. One should note a nonhomogeneous neuronal swelling, weaker in some central domains of the cell body, proportional to the cellular thickness before the hypotonic shock. The hypotonic shock induces a mean integral refractive index decrease. This decrease is consistent with a hypotonic water influx, resulting in a dilution of the intracellular protein concentration, the cellular component which largely determines the mean integral refractive index value.



**Figure 4.** Morphometry of 2 cell bodies before (left panel) and 3 minutes after the onset (middle panel) of a hypotonic shock. Here the  $z$ -axis (cellular thickness) is expressed in micrometers. These values are obtained using the results of the decoupling procedure. Right panel: grey levels-coded distribution of thickness variations resulting from the subtraction of the “standard” image to the “hypotonic” image.

#### **4. Conclusion**

By providing simultaneously interferometric resolution, extremely short measurement time, non-invasiveness, DHM are robust instruments and easy to use instruments which enable to characterize unstained cells for recognition, sorting, static and dynamical analysis. This is illustrated in this paper with phase and thickness measurement of pollen grains and of hypotonic shocks performed on neurones. Real time accurate morphological measurements of living cells has numerous applications in cardiology, immunology, neurobiology, hematology, and in cell physiology and metabolism.

#### **References**

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