

Biological specimen 3D morphology and refractive index separation

The DHM™ technology and a patented decoupling procedure provide, without contrast agents, invasiveness or phototoxicity, the real-time visualization (up to 10'000 fps) of the following characteristics of transparent biological specimens:

- 3D specimen morphometry
- Absorption
- Refractive index related to proteins concentration

By providing together these different parameters, Lyncée Tec provides life sciences with a robust, easy-to-use and versatile instrument to analyze and recognize the structure and dynamics of transparent biological specimens.

The real-time non-invasive quantitative measurement and analysis of the 3D morphology and the refractive index of cells, unicellular organisms or more complex biological specimens has a great importance in visualizing and studying biological processes occurring in living specimens in their natural environment. The measurement of 3D morphology changes can be used for example to assess and quantify cellular swelling resulting from physiological, pathological processes or external stimulation (drugs screening...). The refractive index gives information about the intracellular water fluxes and protein concentration.

Conventional light microscopes, recording only absorption characteristics of transparent biological specimens, are not able to quantify cellular shape and 3D morphology changes. On the other hand, classical contrast enhancing techniques including the widely used phase contrast microscopes (PhC) and/or Nomarski's differential interference contrast (DIC) allow transparent specimen visualization. However they suffer from well-known optical artifacts including halo (PhC) or shading off (DIC) making the interpretation of their images in terms of the corresponding cellular structure elusive.

The DHM™ T1000, based on interferometric

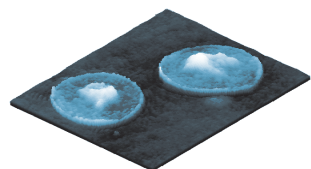
techniques and having the ability to perform an accurate quantitative measurement of the wave front deformations induced by the transparent biological specimen under observation, is an instrument well-suited for life sciences. Combined with a patented decoupling method, DHM™ T1000 performs 3D morphology and refractive index measurement with the features:

- Lateral resolution down to 300nm
- Nanometric thickness resolution (10nm sensibility)
- Mean refractive index along vertical axis
- No contrast agents
- Non-invasiveness
- No phototoxicity (irradiance 200μW/cm²)

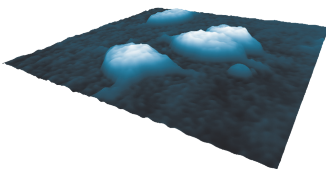
Moreover, digital tools implemented in the Koala Software permit efficient on-line image analysis as well as powerful post-processing of the recorded data such as:

- Temporal monitoring
- Digital focusing: compensation for specimen movement
- Digital compensation of optical aberrations: suppression of misalignment or displacement of the specimen perfusion chamber

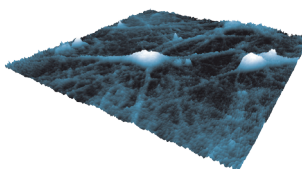
DHM™ technology fits the life sciences needs perfectly by offering a non-invasive instrument for dynamic analysis of living biological specimens: 3D morphology and shape, volume changes, protein concentration and water fluxes amongst others.



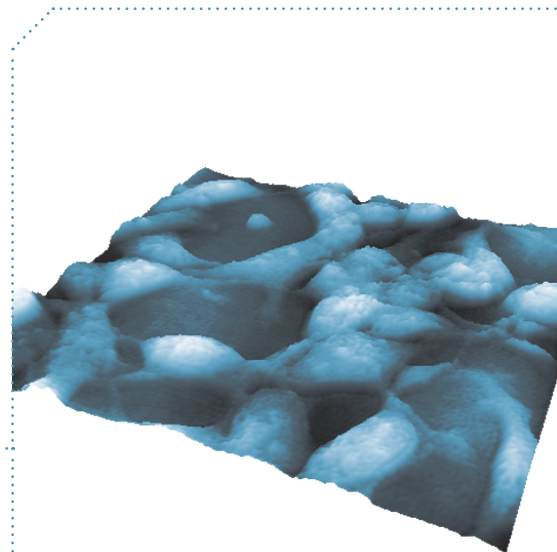
Yew pollen particles
diameter 30μm



Undifferentiated PC12 cell line
field of view 64x64μm²



Cortical mouse neurons net
field of view 100x100μm²



3D visualization of quantitative phase shift of light transmitted through HEK 293 cells obtained with DHM™ T1000.

Quantitative phase contrast

Phase images obtained with DHM™ T1000 contain the spatial distribution of phase delays due to higher speed of light in the perfusion medium compared to the intracellular compartment. Physically, the refractive index $n = c/v$, where c is the speed of light in vacuum and v the speed of light in the medium. The phase (Φ) is related to the optical path length OPL by the relation $OPL = \Phi\lambda/2\pi$, where λ is the wavelength of the microscope light source. In the present case for each pixel i the optical path length is:

$$OPL(i) = (n_{ci} - n_m)h_i + n_m D,$$

where h_i is the cellular thickness corresponding to the pixel i , n_{ci} is the mean value of the intracellular refractive index along the cellular thickness h_i , called the integral refractive index, n_m is the constant refractive index of the perfusion solution and D is the height of the perfusion chamber. The product $n_m D$ is a reference value that can be measured anywhere outside the cell. Monitoring this reference value is important because it enables the compensation of mechanical or thermal instabilities of the set-up during the experiment. For each pixel i , the component of the signal $(n_{ci} - n_m)h_i$, which is specific to the cell, depends on the cell thickness (h_i), the integral refractive index (n_{ci}) and the refractive index of extra-cellular medium (n_m).

Decoupling procedure

A patented method allows the separation of

the measurement of cellular thickness and integral refractive index. It consists in perfusing, consecutively to the standard perfusion solution, a second solution with the same osmolarity (to avoid cell volume variation) but with a different refractive index $n'_m = n_m + \delta n$. The sequence of this procedure is illustrated in Fig. 2. For each pixel i , we obtain two phase recordings ($\Phi_{i,1}$, $\Phi_{i,2}$). The first one is obtained during the perfusion of the standard solution (point 1) and the second one when the decoupling solution has completely replaced the standard solution in the perfusion chamber (point 2):

$$\begin{aligned}\Phi_{i,1} &= 2\pi/\lambda (n_{ci} - n_m)h_i \\ \Phi_{i,2} &= 2\pi/\lambda [n_{ci} - (n_m + \delta n)]h_i.\end{aligned}$$

By solving this equation system we obtain, the integral refractive index n_{ci} , and the cellular thickness h_i for each pixel of the image. Fig. 3 shows an erythrocyte thickness measurement.

Interpretation of thickness and refractive index

The modification of thickness can be interpreted as cell swelling due for example from a hypotonic shock. The refractive index is related to the medium solute that is linearly dependant on the refractive index of the solvent (n_0) and to a specific refraction increment α depending on the nature of the solute:

$$n_{ci} = n_0 + \alpha C.$$

C is the concentration of the solute (principally proteins) and is expressed in terms of mass per unit volume of solution. The measure of the refractive index can therefore provide a good estimation of the concentration of proteins inside the cells by knowing the parameter α . Values of this parameter and more details about this equation can be found in Ref. 2.

Conclusion

The DHM™ T1000 technology is a powerful instrument allowing a non-invasive analysis of biological specimen structure and dynamics providing, thanks to quantitative phase measurements, information concerning both the 3D morphometry and the refractive index related to the protein concentration.

References

1. B. Rappaz et al., "Measurement of the integral refractive index and dynamic cell morphometry of living cells with digital holographic microscopy", *Opt. Express* 13, 9361-9373 (2006)
2. R. Barer, "Refractometry and interferometry of living cells", *J. Opt. Soc. Am.* 47, 545-556 (1957)

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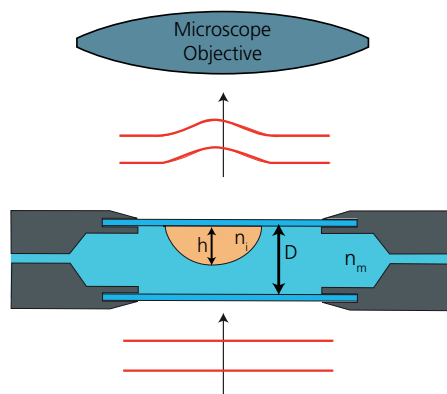


Figure 1: Diagram of the perfusion chamber used to monitor neurons in culture. h_i : cell thickness; n_i : intracellular refractive index; D : perfusion chamber height; n_m : perfusion solution refractive index.

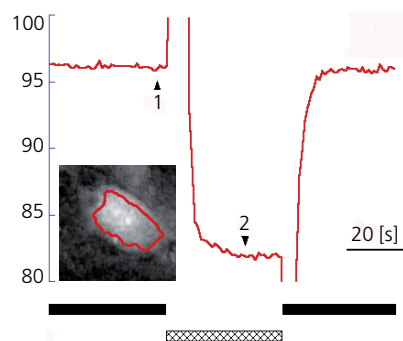


Figure 2: The graphic shows the phase signal (averaged over the surface delineated by the red contour line, inset) plotted versus perfusion time. Rectangles on the bottom indicate: (black) when the standard solution (Mannitol 50mM) or (dashed) the decoupling solution (Nycodenz 50mM) are perfused. Comparison of the phase signal recorded in 1 and 2 allows for the separate measurement of the integral refractive index and the cellular thickness of the specimen.

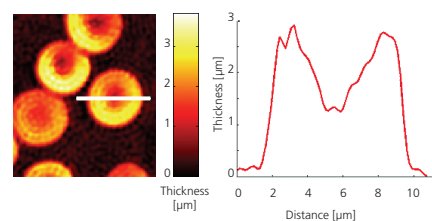


Figure 3: Thickness measurement of Erythrocyte by using the decoupling procedure.