

## Application Note AN03 3D Imaging and Analysis

### Summary

This Application Note illustrates the use of Spatial Light Interference Microscopy (SLIM) for tomographic imaging and 3D analysis of live cells. Tomography of unlabeled live cells is obtained by scanning the focus through the sample, and sequentially taking a stack of image slices along the cell. The obtained stack holds full-3D information of the object, which can be processed further to obtain information regarding the structure and spatial distribution of the object. 3D rendering of the image stack is done on ImageJ to provide a full and flexible view of a cell. Based on the quantitative phase images, this capability expands the applications of SLIM to science and clinic to another dimension.

# INTRODUCTION & PROCEDURE

## Spatial Light Interference Microscopy (SLIM) for 3D imaging

Phi Optics SLIM is a non-invasive phase imaging technology that quantifies the physical properties of live cells and tissues. The output is a live quantitative image (SLIM map) of the specimen on the microscope stage. The intensity of every pixel in the frame is a measure of the optical path length difference (in radians) through the sample, i.e. a phase shift map, which is measured with better than 0.5 nanometers sensitivity [1]. When used with a high NA objective, the white-light illumination of SLIM provides an exceptional optical sectioning effect (Figure 1), which allows for imaging a virtual slice of thickness near 1  $\mu\text{m}$  within the cell [2, 3]. In other words, the specimen is virtually sliced into thin pieces and each slice is imaged sequentially as the imaging system scans its focus through the object. The necessary settings in order to perform a z-stack measurement is integrated in Phi Optics Cell Vista software, which controls both the acquisition and the microscope focus scanning. More detailed description of SLIM can be found in the last page of this note.

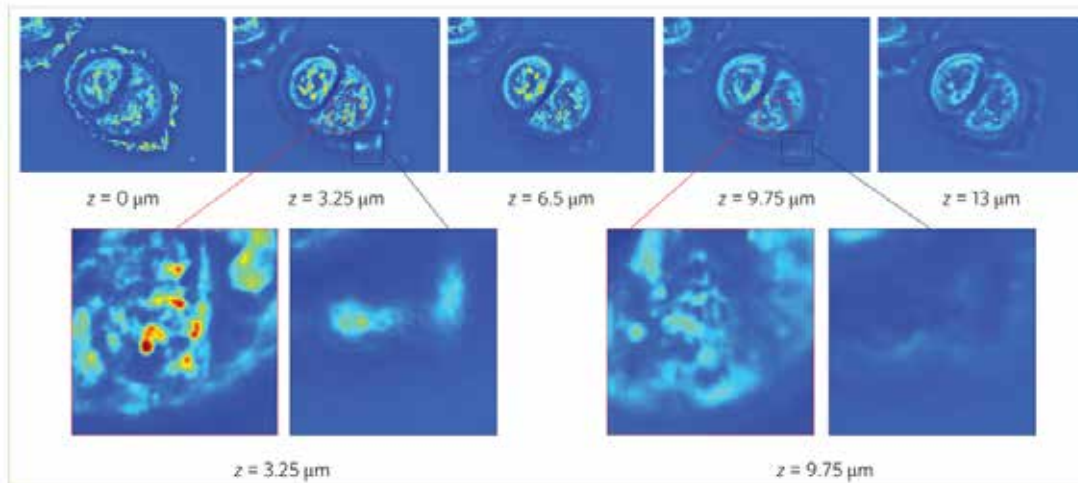


Figure 1. Example of optical sectioning in SLIM is shown as the focus scans through the U2OS cell over a range of 13  $\mu\text{m}$  (top). Red and black outlined regions are zoomed in to more clearly show the optical sectioning ability (bottom). The colour scheme represents the phase value, with red representing large phase values and blue representing small phase values.

## Treating the SLIM z-stack properly

The result of 3D imaging using SLIM is a stack of images scanned through the focus (z-stack). In order to properly analyze the 3D data, the z-stack needs to be adjusted to take into account the spatial scale represented in the stack, i.e. setting the voxel size for the stack. This can be done by adjusting the properties of the stack in ImageJ. The Properties menu can be found under Image menu of the main ImageJ window, and allows the user to set the width, height and depth. Width and height are the spatial scale on the sample plane that a pixel represents in the SLIM map. Depth is simply the z-step size determined by the experiment.

## 3D Rendering and visualizing

ImageJ provides several different plugins to analyze and render the 3D data obtained from SLIM. In this Application Note, four most used plugins found in 3D Analysis and one plugin found under 2D Analysis will be introduced.

### A. 3D Project plugin

3D Project generates a 3D rendering from in the form of varying perspectives along the vertical axis. In other words, 3D Project generates 360° rotation of the reconstructed object. The result is a stack of images, each showing a projection of the object at the angle corresponding to the image (Figure 2). The projection modes are: nearest point (shows the closest voxel along the line of sight), brightest point (shows the brightest voxel along the line of sight), and mean value (shows the average value of the voxels along the line of sight). The angle increment, and upper and lower threshold for transparency can be determined. 3D Project also provides an option to interpolate, in order to make the rendering from the discrete data more seamless. This plugin requires the stack to be converted to 8-bit.

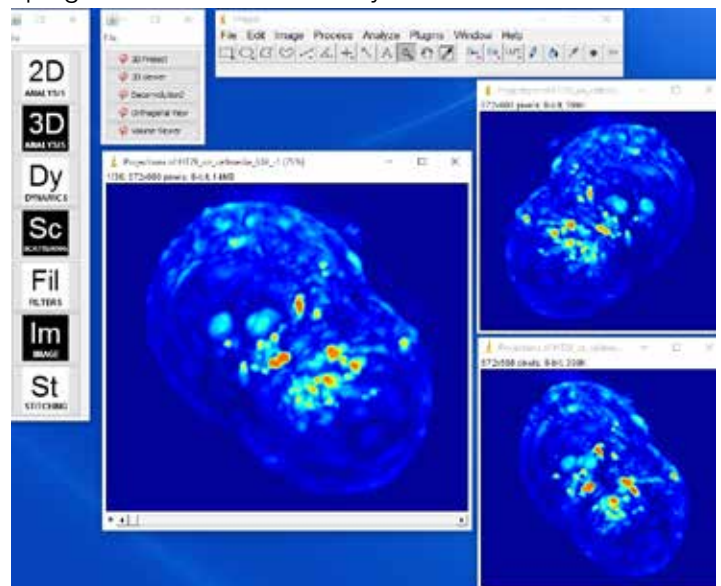


Figure 2. Result from 3D Project plugin

## B. 3D Viewer plugin

3D Viewer constructs a volume from a 3D stack. Unlike in the 3D Project, the user can rotate the volume freely without being constrained by one axis and zoom in and out (Figure 3). The threshold for transparency can also be applied under the “Edit” menu of the result window. Moreover, the plugin allows the user to input multiple channels for reconstruction, allowing an overlay between multiple imaging modalities.

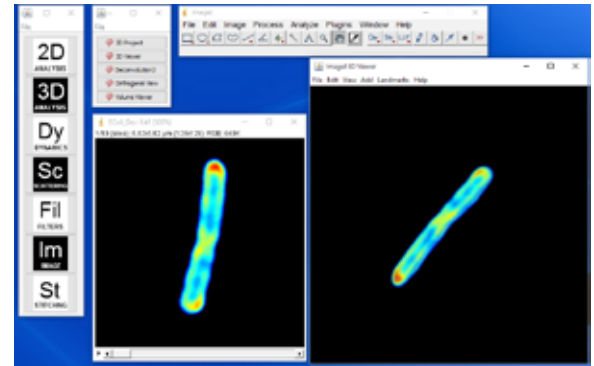


Figure 3. Input stack and result of 3D Viewer plugin

## C. Orthogonal View plugin

Orthogonal View shows three cross-sections along the three representative planes, x-y, y-z, and x-z, taken at a specified location. The plugin opens two additional windows around the original stack to represent the two orthogonal views of the stack (Figure 4). The location of cross-section is indicated by yellow lines on the images, and can be selected by simply clicking on the image or by scanning the z-stack.

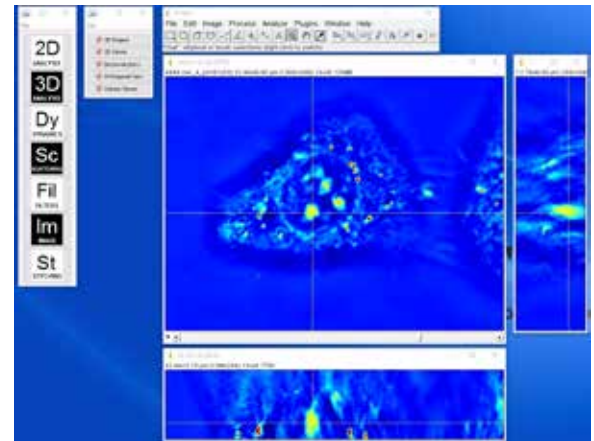


Figure 4. Result of Orthogonal View plugin

## D. Volume Viewer plugin

Volume Viewer provides 3D rendering of a stack and also shows the cross-sections along x-y, y-z, x-z planes. There are several modes of operation: Slice, Slice & Borders, Max Projection, Projection and Volume. Slice and Slice & Borders show one slice orthogonal to the line of sight of the user. Max Projection and Projection shows one perspective which represents the maximum or the average values along the line of sight. Volume constructs a 3D object from the stack, similarly to the 3D viewer (Figure 5). The user can adjust the alpha, or the transparency, of the whole stack easily by drawing an own curve on the histogram located on the right side of the result window.

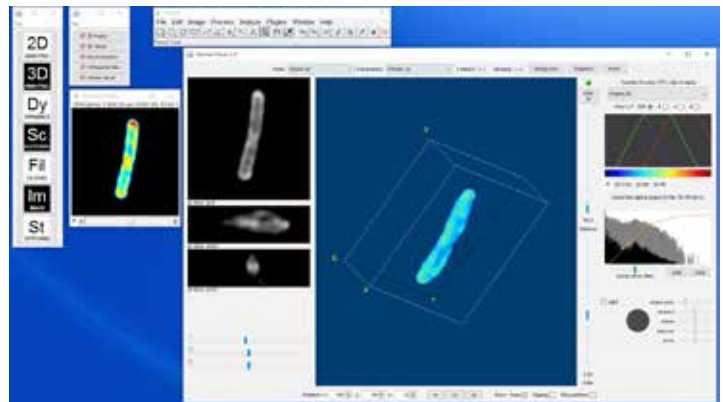


Figure 5. Input stack and result of Volume Viewer in “Volume” mode

## E. Cell Volume plugin

Although one can certainly use the 3D reconstruction to determine the cell volume, SLIM provides an easy method for determining the volume of a cell when used with a low NA objective. Using its capability of inferring the thickness of the specimen, the volume at each pixel can be calculated, and by integrating these volumes over the area covered by the cell, the cell volume can be determined. Phi Optics also provide an ImageJ plugin that calculates the cell volume from the phase SLIM map, by taking the pixel size, and refractive indices of the cell and the background as inputs (Figure 6). The plugin can be found under 2D analysis menu.

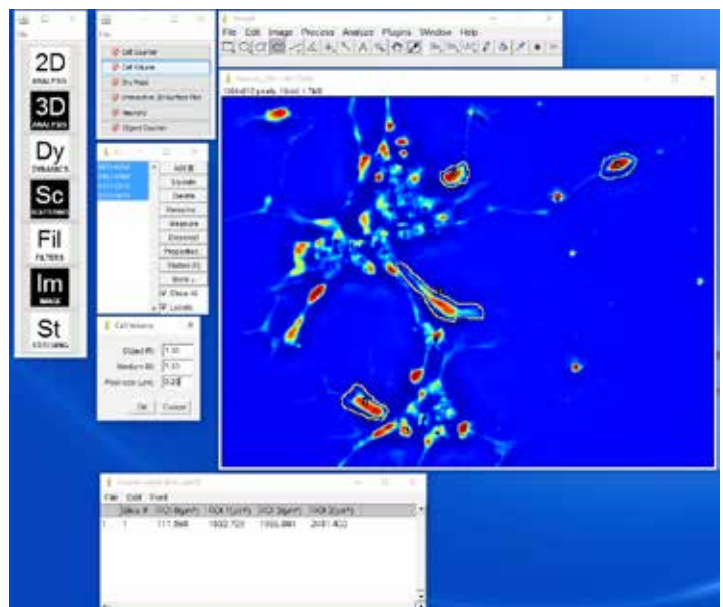


Figure 6. Cell volume plugin and results for multiple ROIs



## Deconvolution: White-light Diffraction Tomography

In order to improve the image quality and resolution, the 3D stack can be deconvolved using the system point spread function (PSF), which can be calculated or measured using a small bead [2,3]. The result yields suppressed out-of-focus lights and better resolution, and thus, allows the user to see smaller details of the specimen (Figure 7 and Figure 8). Deconvolution can be performed using the DeconvolutionJ plugin under 3D Analysis menu. Two tomograms obtained by SLIM with deconvolution are introduced below.

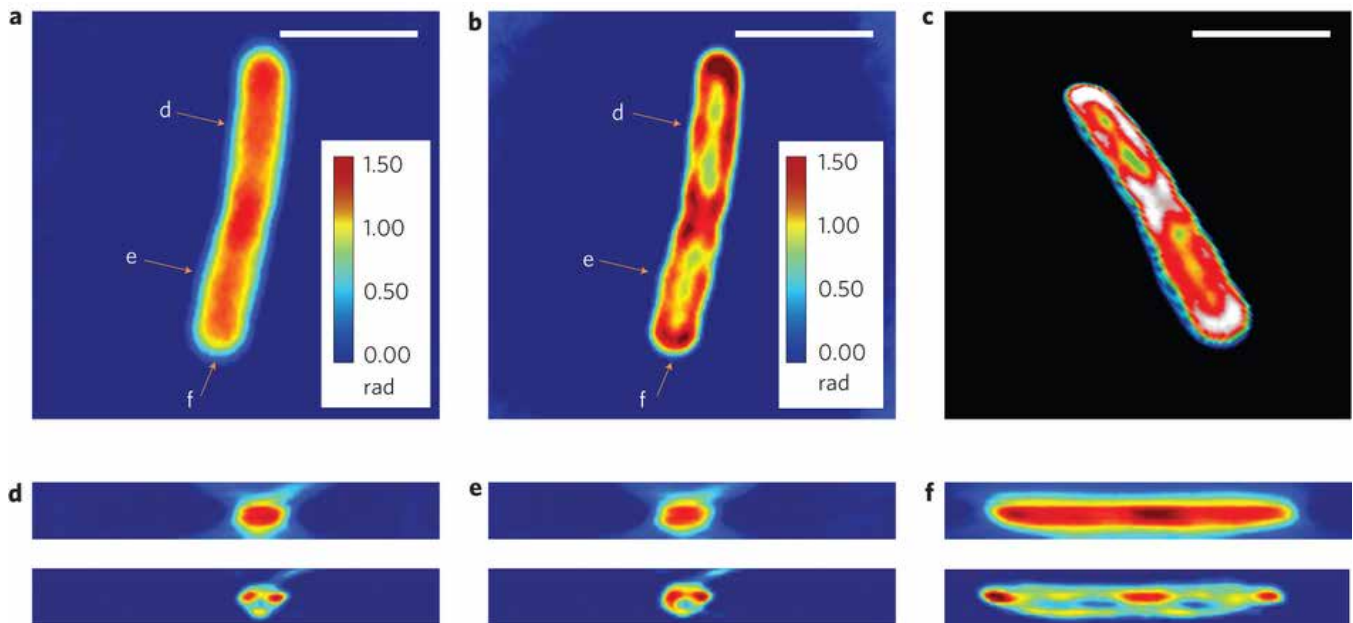


Figure 7. WDT of *E. coli* cells. (a) The centre frame of a z-stack measurement using a  $\times 63/1.4$  NA oil immersion objective. (b) Deconvolution result of the same z-slice as in a, clearly showing a resolved helical structure. (c) Centre cut of the three-dimensional rendering of the deconvolved z-stack, which shows both the overall cylindrical morphology and a helical subcellular structure. (d–f) Cross-sections of the measured z-stack (top row) and the deconvolved z-stack (bottom row). Each figure label corresponds to the markers shown in a, b and is in the same scale as a, b. Scale bars, 2mm. A z-stack of 17 images, each with  $128 \times 128$  pixels is used for the reconstruction, which requires about 3 min for sparse deconvolution

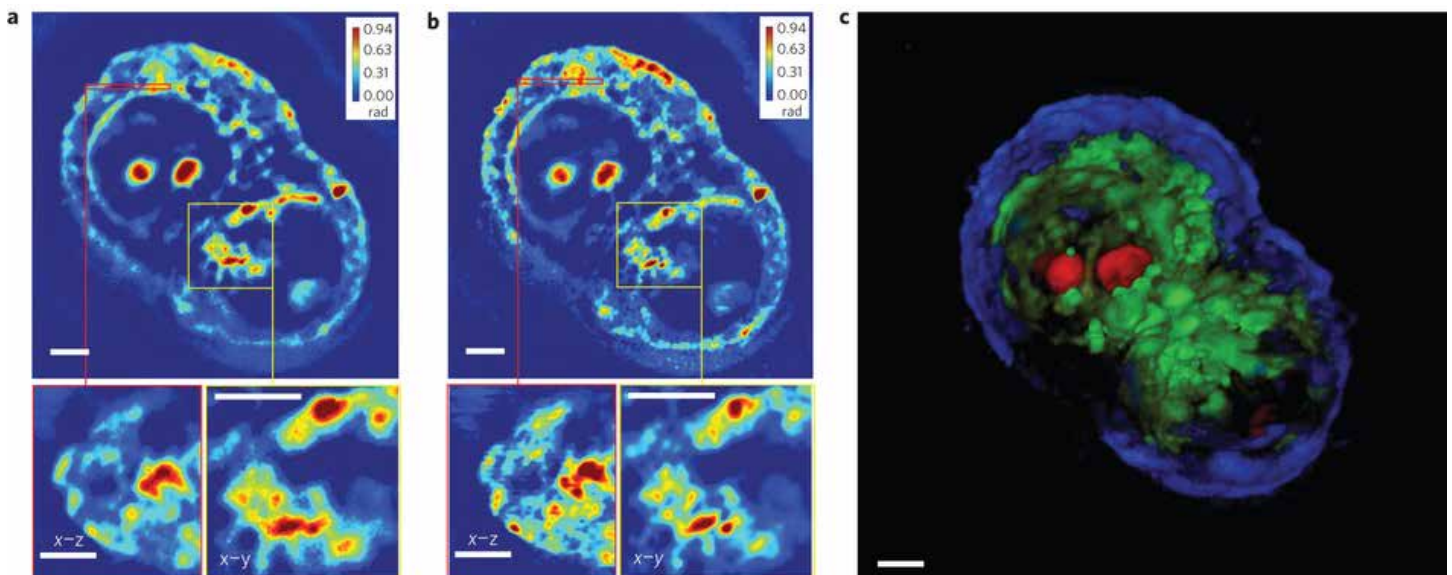


Figure 8. (a) A measured z-slice (top), a cross-section at the area indicated by the red box (bottom left) and a zoomed-in image of the area indicated by the yellow box (bottom right), measured using a  $\times 63/1.4$  NA oil immersion objective. (b) A deconvolved z-slice corresponding to the measurement shown in a (top), a cross-section at the area indicated by the red box (bottom left) and a zoomed-in image of the area indicated by the yellow box (bottom right). By comparing a and b, the resolution increase can be clearly seen. (c) False-colour three-dimensional rendering of the deconvolution result (Supplementary Movie 3). We used z-stacks of 140 images, each with a dimension of  $640 \times 640$ . Owing to the large image dimension, the image is split into 25 sub-images for faster deconvolution. Overall, the deconvolution process took approximately an hour. Scale bars in all panels, 5  $\mu\text{m}$ .

## References

- [1] G. Popescu (2011) Quantitative phase imaging of cells and tissues (McGraw-Hill, New York)
- [2] T. Kim, R. Zhou, M. Mir, S. D. Babacan, P. S. Carney, L. L. Goddard and G. Popescu, Nature Photonics, 8, 256-263 (2014)
- [3] M. Mir, S. D. Babacan, M. Bednarz, M. N. Do, I. Golding and G. Popescu, PLoS ONE, 7 (6), e38916 (2012)

# SPATIAL LIGHT INTERFERENCE MICROSCOPY (SLIM)

Phi Optics patented SLIM technology employs optical interferometry for extreme sensitivity to structure and dynamics. Phi Optics implements SLIM as an add-on to all major brand optical microscopes (10X to 100X magnifications) (see Figure 9). The SLIM approach to quantitative phase imaging provides speckle-free images due to high sensitivity of the measurement (nanometer scale spatial noise). Submicron optical sectioning is facilitated by high NA objectives and the micron-scale coherence length of the illumination - SLIM can render 3D tomographic images of transparent structures just by scanning the specimen through focus (Z-scanning). The design modularity enables multiplexing with fluorescence imaging for multimodal, in-depth biological studies.



Figure 9. Phi Optics SLIM module attached to a ZEISS Axio Observer Z1 microscope

Imaging live cells using classical brightfield microscopy is notoriously difficult because they absorb and scatter very little light. Fluorescence microscopy employs fluorophores which absorb and emit light, rendering the cells visible. The fluorophores can be genetically encoded or injected into the live cells and their location and emission intensity is used to accurately quantify the cell features and processes. Continuous imaging is possible only for short periods of time (time-lapse) to avoid phototoxicity, photo bleaching and measurement bias. Live unstained cells exhibit gradients of optical path length (i.e. product of thickness and refractive index) across their structure. Phase contrast (PC) and differential interference microscopy (DIC) modalities to convert minute changes in optical path length into differences in brightness (amplitude contrast). These techniques are not intrinsically quantitative or specific, and lack the resolution of fluorescence microscopy but continuous long term imaging is possible because of the low levels of illumination required.

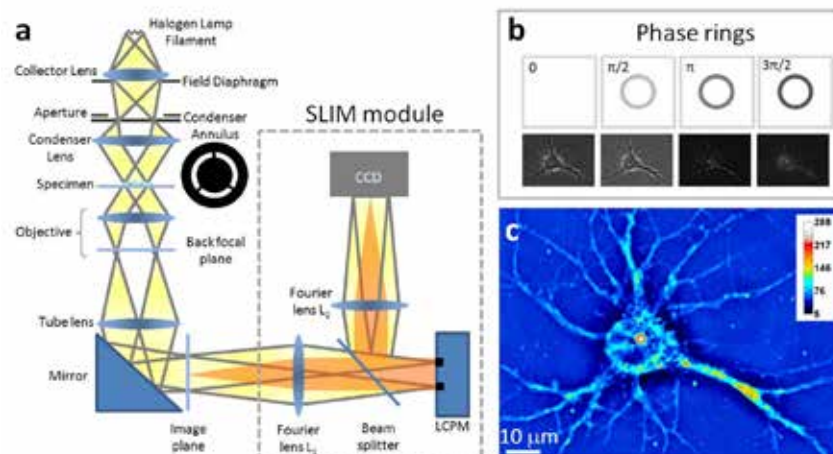


Figure 10. SLIM principle. (a) Schematic setup for SLIM. The SLIM module is attached to a commercial phase contrast microscope (b) The phase rings and their corresponding images recorded by the CCD. (c) SLIM quantitative phase image of a hippocampal neuron.

Phi Optics SLIM is a non-invasive phase imaging technology that quantifies the optical path length differences in a biospecimen and converts them into thickness, dry mass area density and refractive index maps. Figure 10 illustrates the principle of technology in its reference implementation. A live cell in culture medium is imaged with the phase contrast modality of the microscope: the light passing through the object (scattered beam) and the light passing through the medium (reference beam) combine through interference in the image plane. The optical path length differences between the beams (i.e. the phase shift) in each point of the image plane are converted into brightness differences. Optically dense areas of the cell (e.g. nucleus) introduce a phase shift of up to  $-0.5\pi$  radians in the scattered beam with respect to the reference beam. In the most common phase contrast implementation, before reaching the image plane the reference beam also undergoes a  $+0.5\pi$  radians phase shift by passing through a phase plate: a glass ring with finite thickness in the back focal plane of the objective. Destructive interference generates a dark image for the dense portions of the cell with respect to the grey background (see Figure 10b).

The SLIM module relays the image plane with minimal aberrations (diffraction limited) at a 1:1 ratio to a camera sitting at its exit port. The active element at the heart of the SLIM module is a liquid crystal spatial light modulator (SLM). The SLM is conjugated with the back focal plane of the microscope objective, and it modulates the reference beam like a phase plate with variable thickness. To create a quantitative phase image the SLM shifts the phase of the reference beam by a fixed amount ( $0, 0.5\pi, \pi, 1.5\pi$ ) and the camera captures the resulting frame (Figure 10b). The CellVista software module combines the four frames by solving the field interference equations in each point of the frame – the result (Figure 10c) is a quantitative-phase (SLIM) image that is uniquely determined.

SLIM is a wide field quantitative imaging method thus it can measure simultaneously large populations of cells at full camera resolution (e.g. 2 mm FOV for 10X objective at 4.2 MP camera resolution). Wide field optical sectioning (e.g. 850 nm Z-resolution for 100X/1.4NA objective) enables 3D tomography. All microscope output is acquired with the same camera which enables seamless overlay of SLIM images with fluorescence channels.