

## Application Note AN01 Cell Growth and Proliferation

### Summary

This Application Note illustrates the use of Spatial Light Interference Microscopy (SLIM) to measure the dry mass of many individual adherent cells in various conditions, over spatial scales from micrometers to millimeters, temporal scales ranging from seconds to days, and cell types ranging from bacteria to mammalian cells. Combining SLIM with fluorescence imaging provides a unique method for studying cell cycle-dependent growth: the fluorescent reporter indicates the cell growth stage and SLIM provides non-invasive measurements of cell dry mass with single cell resolution (femtogram sensitivity). This has clear applications in areas such as **oncology**, **developmental biology** and in **drug discovery/safety**.

# INTRODUCTION & PROCEDURE

## Spatial Light Interference Microscopy (SLIM)

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. The intensity of every pixel in the frame is a measure of a phase shift map (in radians) or the optical path length difference (in nanometers) through the sample, which is measured with sensitivity better than 0.5 nanometers [1]. As shown in Figure 1, the phase shift map is converted on-the-fly to other SLIM maps, with their respective pixel intensity: thickness (in microns), dry mass area density (picograms per square micron) and refractive index. More detailed description of SLIM can be found in the last page of this note.

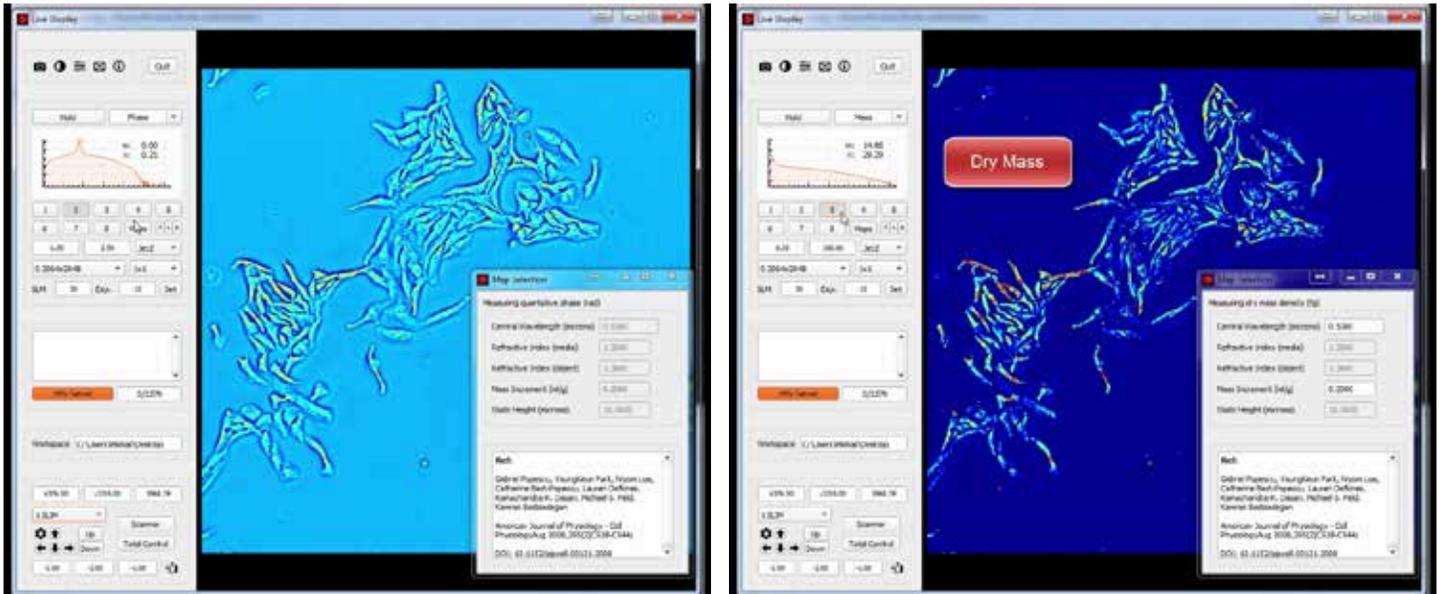


Figure 1. CellVista software working in phase measurement mode (in radians) and in dry mass measurement mode (in picograms). The setting can be changed in the Map Selection menu.

## Procedure to extract dry mass

The measured path length information at each pixel is directly related to the dry mass density at that point, and, thus, by integrating over a Region of Interest (ROI), the total dry mass in the ROI is measured. When translated into dry mass, SLIM's sensitivity corresponds to changes on the order of femtograms [2]. Phi Optics provides a set of plugins based on the NIH ImageJ for image post-processing; the analysis can be done on any of the two types of acquired SLIM maps. To extract cell dry mass, the steps are:

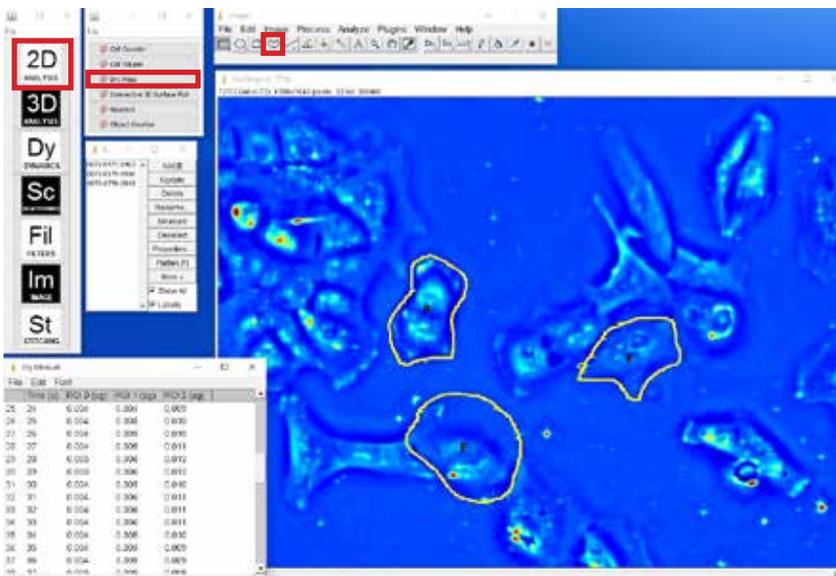


Figure 2. Dry Mass plugin for multiple ROIs. For the three ROIs indicated on the SLIM map, the total dry mass within the field of view for each frame is obtained as shown in the result table in the bottom left corner.

1. Load a SLIM map or a time series (either in phase or dry mass) in ImageJ
2. ROIs containing the object to be measured can be selected using the freehand selections tool and added to the ROI manager
3. Select the Dry Mass plugin in 2D Analysis menu
4. For each frame, the total dry mass in each ROI is displayed in a separate column in the measurements window.
5. The result of dry mass measurement can be saved into a .xls file for further plotting and analysis.

## Single cell growth measurement

SLIM was used to measure the dry mass of individual E-coli cells as shown in Figure 3 [2]. The measurements were performed simultaneously on many individual cells and relevant statistics were acquired on large populations.

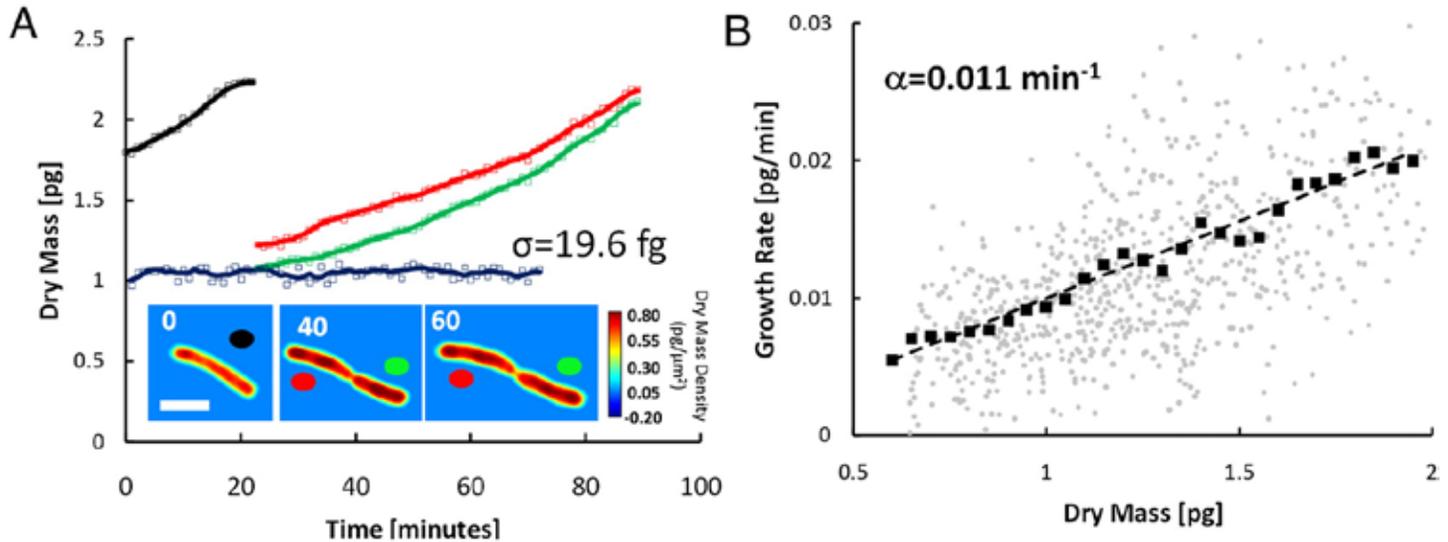


Figure 3. SLIM measurements of E. coli growth. (A) Dry mass vs. time for a cell family. Growth curves for each cell are indicated by the colored circles on the images. Images show single cell dry mass density maps at the indicated time points (in minutes). (Scale bar: 2  $\mu\text{m}$ .) The blue line is a fixed cell measurement, with SD of 19.6 fg. Markers indicate raw data, and solid lines indicate averaged data. (B) Growth rate vs. mass of 20 cells measured in the same manner. Faint circles indicate single data points from individual cell growth curves, dark squares show the average, and the dashed line is a linear fit through the averaged data; the slope of this line, 0.011  $\text{min}^{-1}$ , is a measure of the average growth constant for this population. The linear relationship between the growth rate and mass indicates that, on average, E. coli cells exhibit exponential growth behavior [2].

## Simultaneous SLIM and fluorescence imaging

To study single cell growth in an asynchronous culture and obtain information about cell cycle-dependent growth, SLIM was combined with epi-fluorescence imaging as shown in Figure 4 [2]. While SLIM measures the dry mass at each point in time, the fluorescence markers indicated the phase of the cell within the cell-cycle. **SLIM shares the same optical path with all of the other channels of the microscope, enabling seamless overlay of all normal microscope modalities. The one-to-one correspondence between the cells in SLIM images and the cells in fluorescence images enables cycle-dependent growth measurements.**

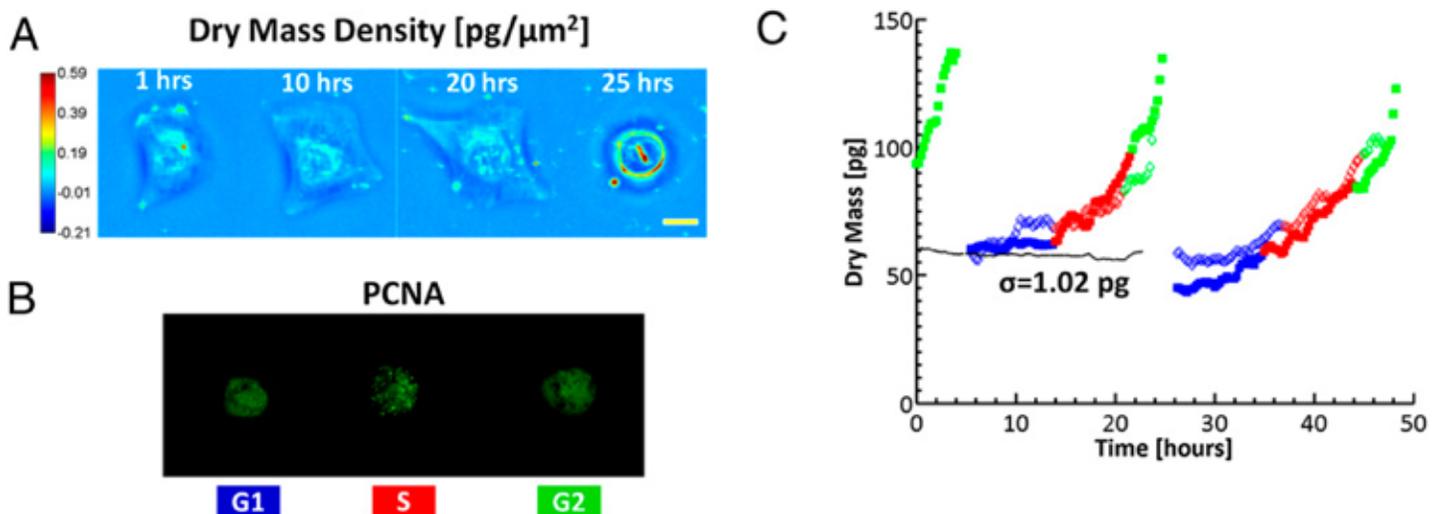


Figure 4. SLIM measurement of U2OS growth over 2 d. (A) Dry mass density maps of a single U2OS cell over its entire cycle at the times indicated. (Scale bar: 25  $\mu\text{m}$ .) Color bar indicates dry mass density in  $\text{pg}/\mu\text{m}^2$ . (B) Simultaneously acquired GFP fluorescence images indicating PCNA activity; the distinct GFP signal during S phase and the morphological changes during mitosis allow for determination of the cell cycle phase. (C) Dry mass vs. time for a cell family (i.e., 1  $\rightarrow$  2  $\rightarrow$  4 cells). The two different daughter cell lineages are differentiated by the filled and open markers; only one daughter cell from each parent is shown for clarity. Different colors indicate the cell cycle as reported by the GFP-PCNA fluorescence. The dotted black line shows measurements from a fixed cell, which has SD of 1.02 pg [2].

## Measurement of growth in large populations

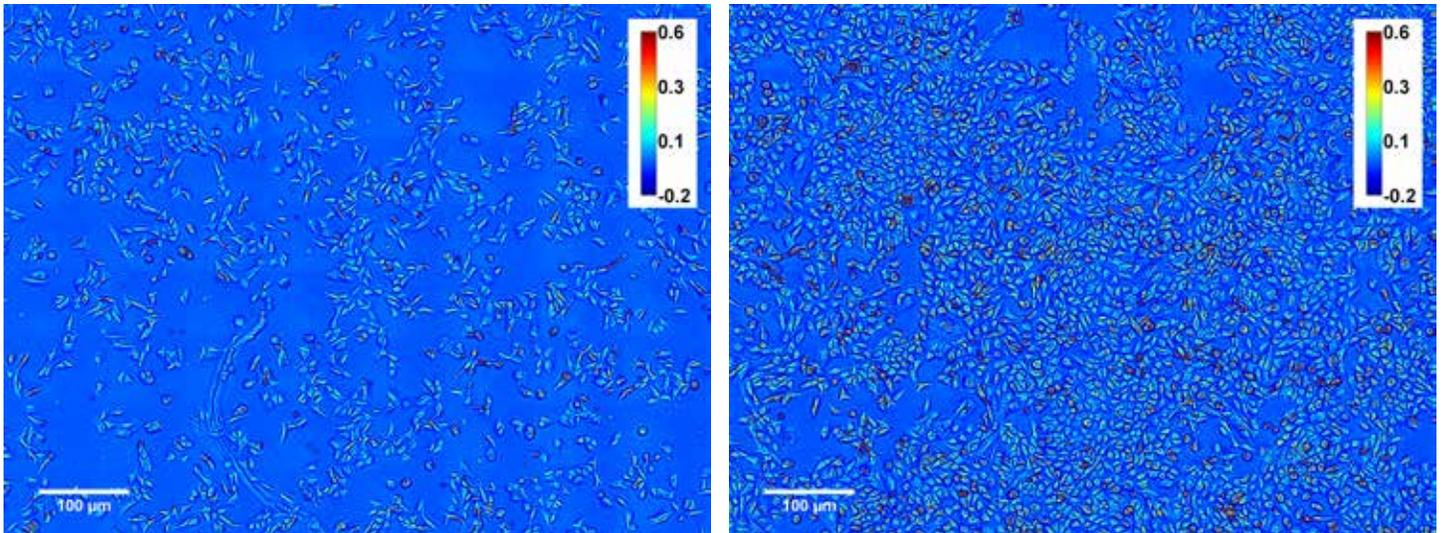
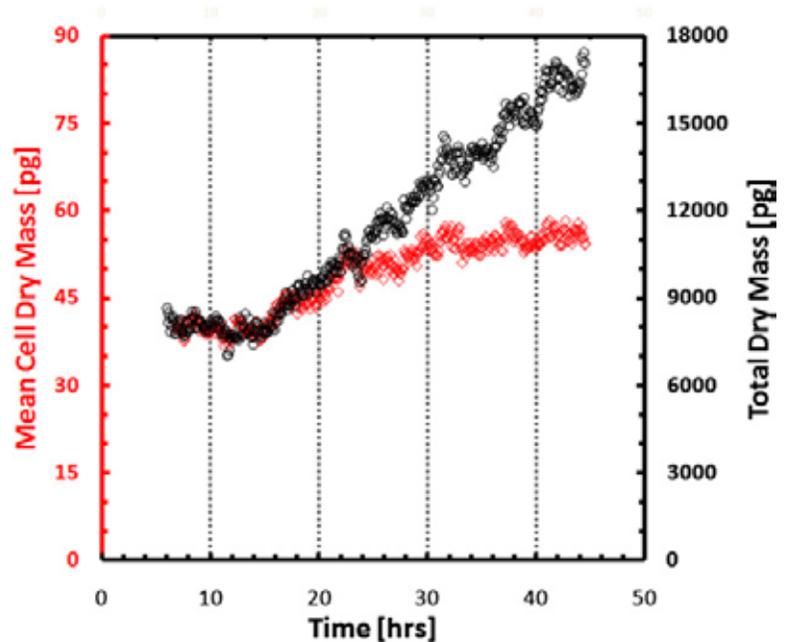


Figure 5. SLIM measurement of synchronized U2OS cell culture over more than 2 days. Black shows dry mass vs. time for a synchronized cell population over a  $3.2 \times 2.4$  sq. millimeters field of view obtained by montaging  $8 \times 8$  microscope images. (10 $\times$  objective, NA = 0.3). Red shows cell mean dry mass vs. time. Images show the field of view at 4 (left) and 45 h (right). Color bar is in radians (phase shift) [2].

Because of the large resolution of SLIM frames (e.g. 4 megapixels), large cell populations can be studied. SLIM was tested in cell growth measurements of mammalian cells over more than one cell cycle as shown in Figure 5 [2]. The results show that the mean cell mass evolves synchronously in time with the total mass of the entire population during the duration of a (mean) cell cycle, i.e., 22–26 h, after which it levels off. This finding indicates that after one cell cycle, the culture loses synchrony and the single cell mass is limited by mitosis.



In conclusion, the results presented here establish that SLIM provides a number of advances with respect to existing methods for quantifying cell growth: (i) SLIM can perform parallel growth measurements on an ensemble of individual cells simultaneously; (ii) spatial and temporal correlations, such as cell–cell interactions, can be explored on large scales; (iii) in combination with fluorescence, specific chemical processes may be probed simultaneously; (iv) the environment is fully biocompatible and identical to widely used equipment; (v) the imaging nature of SLIM offers the ability to directly monitor cells and their surroundings, elucidating the nature of any artifacts and providing morphological information simultaneously; (vi) a lineage study is possible, i.e., a cell and its progeny may be followed; and (vii) measurements can be performed on cells ranging from bacteria to mammalian cells.

## References

- [1] G. Popescu (2011) Quantitative phase imaging of cells and tissues (McGraw-Hill, New York) p. 313.
- [2] M. Mir, Z. Wang, Z. Shen, M. Bednarz, R. Bashir, I. Golding, S. Prasanth and G. Popescu, Optical Measurement of cycle-dependent growth, *Proc. Natl. Acad. Sci.*, 108 (32), 13124 (2011).
- [3] M. Mir, T. Kim, A. Majumder, M. Xiang, R. Wang, S. C. Liu, M. U. Gillette, S. Stice and G. Popescu, Label-free characterization of emerging human neuronal networks, *Scientific Reports*, 4, 4434 (2014)
- [4] M. Mir, A. Bergamaschi, B. S. Katzenellenbogen and G. Popescu, Highly sensitive quantitative imaging for monitoring single cancer cell growth kinetics and drug response, *PLoS ONE*, 9 (2), e89000 (2014)

# 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 6). 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 6. 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.

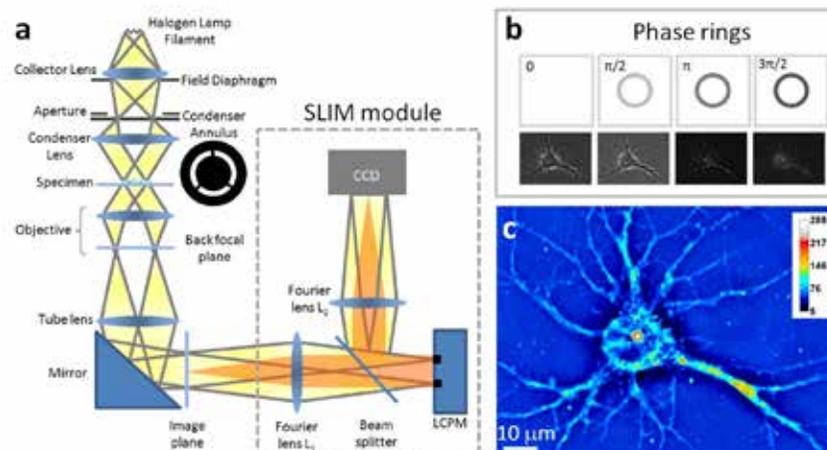


Figure 7. SLIM principle. (a) Schematic setup for SLIM. The SLIM module is attached to a commercial phase contrast microscope (b) The phase rings and their corresponding images recorded by the CCD. (c) SLIM quantitative phase image of a hippocampal neuron.

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 7 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 7b).

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 7b). The CellVista software module combines the four frames by solving the field interference equations in each point of the frame – the result (Figure 7c) 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.