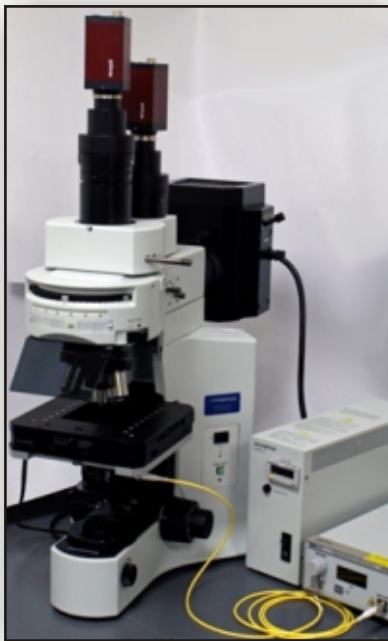


## *In-situ* stain-free imaging of live cells with the Phase Focus Virtual Lens®

In this note we discuss the application of the Phase Focus Virtual Lens® to high contrast stain-free cell microscopy. We demonstrate imaging of immersed cells in deep containers such as petri-dishes, multi well plates and flasks. We also demonstrate the ease with which post-acquisition focussing, and segmentation via simple thresholding, can be performed.

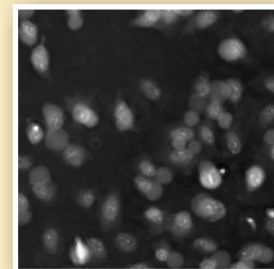
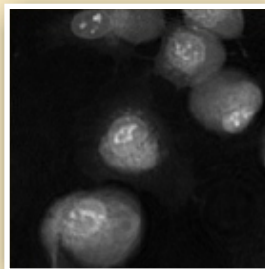


Improved sensors for live cell analysis are needed that can generate high contrast images without staining; that can provide improved segmentation and feature extraction; that enable auto-focussing without increased light dose and instrumental complexity; and that lend themselves to automated data collection and analysis, all over prolonged periods of time<sup>1,2</sup>.

Phase Focus has developed a new technology, the Phase Focus Virtual Lens®, that replaces or complements a conventional microscope lens system with a computer program. This not only eliminates the restrictions and performance limitations of today's conventional lenses, but also enables a range of new and improved applications.

The Virtual Lens can be integrated in a straightforward manner with existing instrumentation such as conventional microscopes or high content screening systems.

**Figure 1:** Typical Phase Focus Virtual Lens phase images of fixed A549 cells in a phosphate buffer. The unstained cells can be seen with very clear contrast and without the halo effect usually associated with conventional phase contrast techniques.



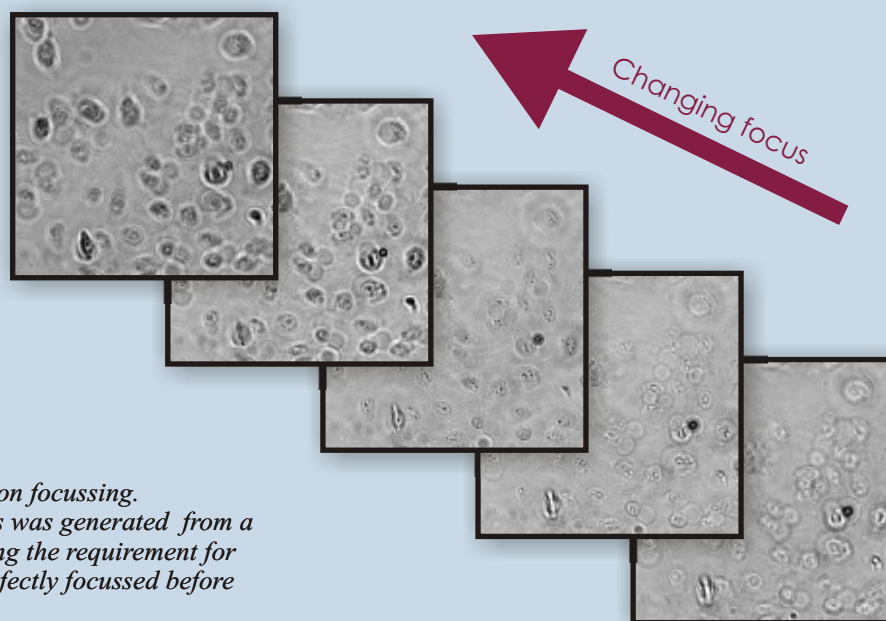
1. Therapeutic Areas and Research Technologies: Areas of Interest, Merck & Co., Inc., June 2008

2. Thomas, N. High-Content Screening: A Decade of Evolution. *Journal of Biomolecular Screening* 15: 1-9, 2010.

The Virtual Lens brings a unique combination of benefits to cell imaging:

- High contrast without stains
- Free of conventional distortions
  - No conventional phase contrast microscopy artefacts
  - No lens-associated aberrations
- Wide variety of non-contact geometries. E.g.:
  - Fully lensless near-field imaging
  - Lens-assisted imaging with very large working distance ( $\geq 30$  mm)
- Large fields of view within extended specimens
  - User-defined; independent of resolution
- Perfect focus every time
  - Post-acquisition through-focal series reconstruction and/or interactive focal plane selection
- Cell characterisation and feature extraction
  - Quantification of cell thickness; shape; refractive index
  - Simple segmentation
- Extremely easy to use
- Operator-invariant results

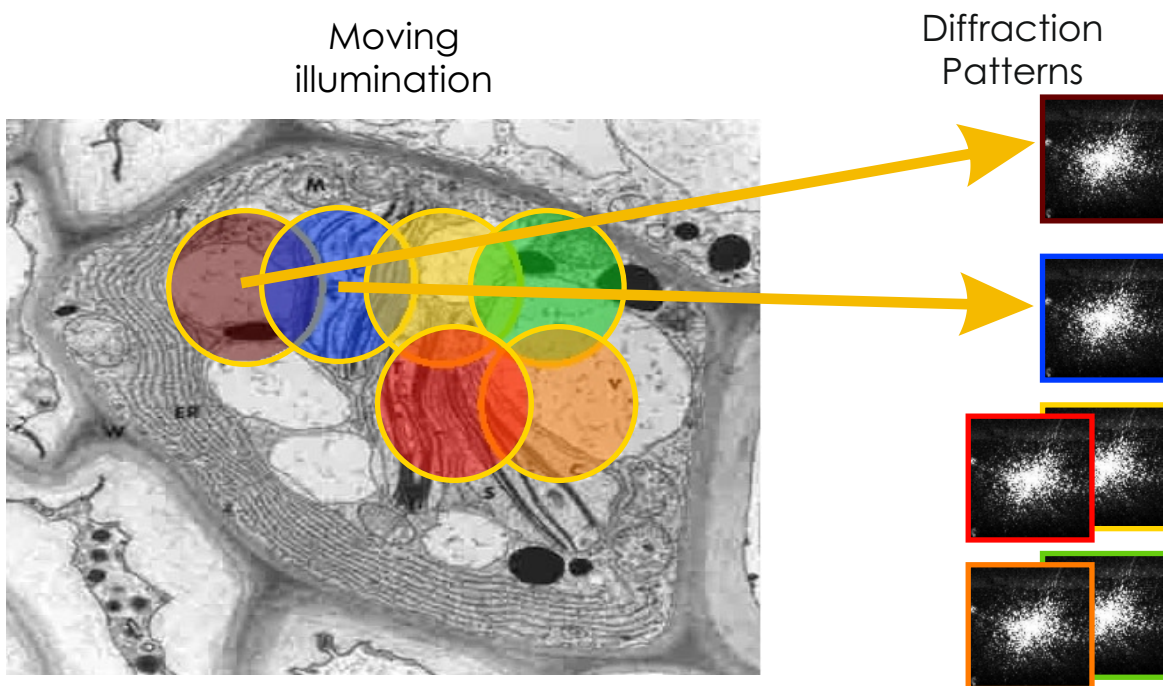
While the Phase Focus Virtual Lens typically operates in transmission mode for cell imaging and other life science applications, it has also been demonstrated in reflection mode for a wide variety of materials science and metrology applications. (For further information, see App Note AN04.)



**Figure 2:** Post-acquisition focussing. This through-focal series was generated from a single dataset, eliminating the requirement for the microscope to be perfectly focussed before data collection.

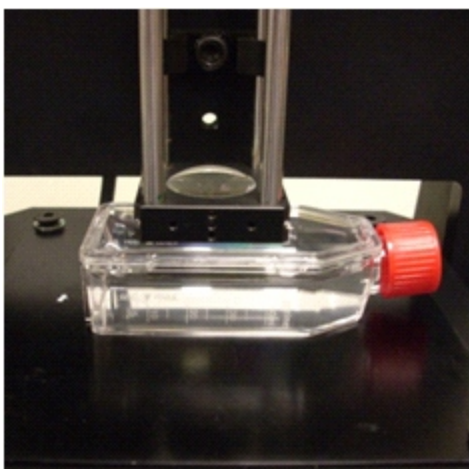
## Principle of Operation

An optical wavefront contains both amplitude and phase information. However, because detectors such as the eye or a CCD camera can record only intensity, the phase information in a conventional microscopy experiment is lost. The power of the Virtual Lens is that it is able to recover this lost phase information, knowledge of which enables many important benefits. Firstly, an image of the specimen can be reconstructed in the computer from the diffraction patterns, without the need for any lenses. Secondly, having reconstructed the entire wavefront, focussing at any plane becomes possible after data acquisition is complete. And thirdly, two images are routinely obtained at any chosen focal plane: one (the amplitude image) is essentially equivalent to the conventional brightfield image; but the other (the phase image) is a quantitative map that shows how by how much the phase of the illumination has been retarded as it passes through (or is reflected by) the specimen. This quantitative phase information has multiple benefits in cell imaging. For example, it provides high contrast in otherwise transparent specimens, and it enables measurement and extraction of specimen features such as thickness or refractive index. Importantly, the Virtual Lens phase image has none of the artefacts traditionally associated with qualitative phase contrast methods such as Zernike and differential interference contrast. As such, it is very "clean" and much more amenable to automated post processing such as segmentation and feature extraction. For a more detailed explanation of the Phase Focus Virtual Lens technology, please refer to "TB01 - The Phase Focus Virtual Lens<sup>®</sup>"



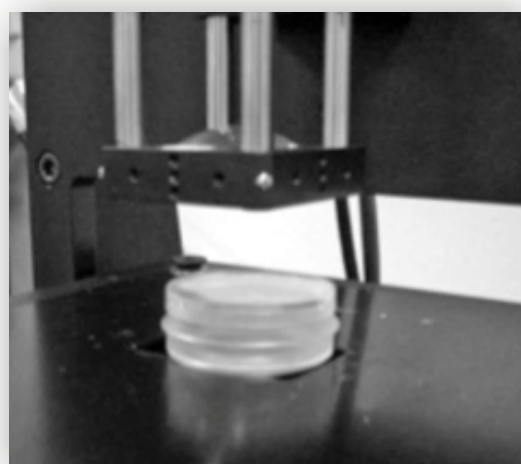
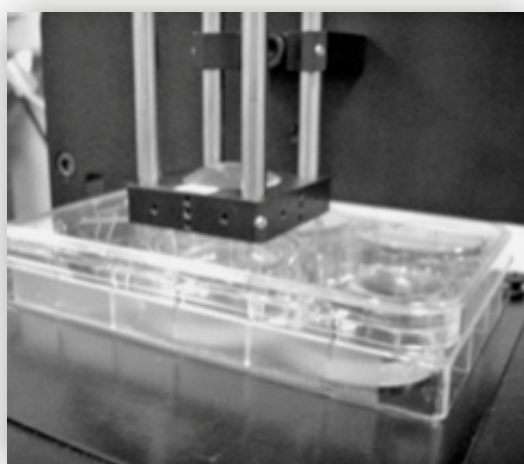
**Figure 3:** Data collection: a series of overlapping regions, covering a user-defined area of interest, is illuminated and the corresponding diffraction patterns recorded on a standard detector (such as a CCD camera).

## Lens-assisted large working distance option



The resolution of a microscope is determined by the wavelength of radiation used and the effective numerical aperture (NA) of the condenser and objective lenses. This is because high-resolution features in the object scatter waves to large angles. One problem with conventional very high resolution microscopy is that the objective lens must be mounted close to the object in order to capture these high resolution data. In the case of the Virtual Lens, resolution is determined by the angle subtended by the detector at the object plane; a large detector can be placed quite some distance downstream of the object, yet still capture a high range of scattering angles, and hence retain good resolution without requiring a short working distance.

In practice, a very large working distance (>30 mm) is achieved without a major compromise of resolution by operating the Virtual Lens in a "lens-assisted" configuration: A large "poor" lens (e.g.: a simple achromatic doublet), mounted downstream of the object, is used to tailor the demagnification of the diffraction patterns so that they are matched to the CCD size. Unlike an imaging lens, this lens is not required to interfere accurately beams that pass through opposite edges of it; it is only required to interfere narrowly spaced rays. In this configuration, large aberrations in the lens can be tolerated because they only cause geometric distortion of the diffraction pattern, which can be easily removed computationally. Of course, high-performance conventional objective lenses may still be used if desired - for example, to integrate or compare the Virtual Lens features with conventional techniques.



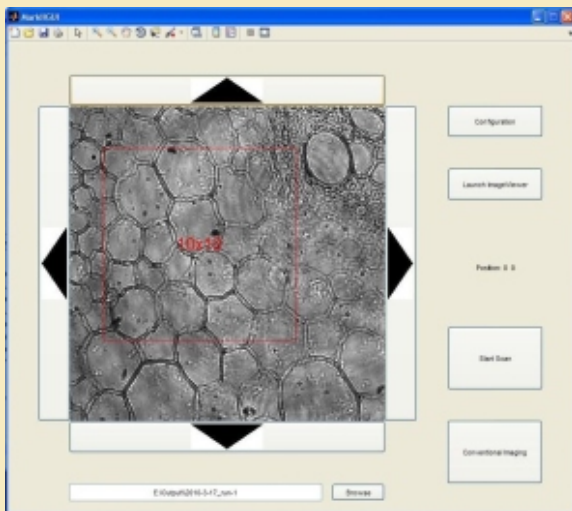
**Figure 4:** The very large working distances available with the Virtual Lens enable imaging of cells situated within deep flasks, petri dishes and multi-well plates.

## High contrast stain-free imaging

### I. Ease of Use and Operator Invariance

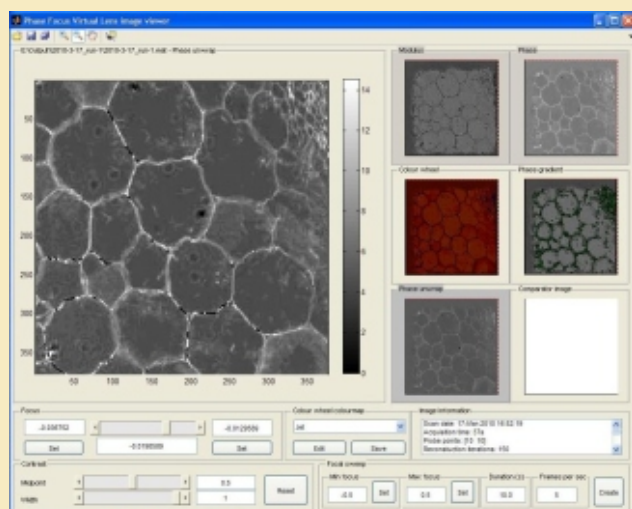
Because the reconstructed amplitude and phase images are both quantitative, they reflect properties inherent to the specimen rather than operator- or instrument-dependent factors. Thus, results are not dependent upon subjective operator interventions, and repeated experiments are highly consistent. This supports the integration of the Virtual Lens methodology into automated process work flows.

Moreover, data acquisition and reconstruction is an extremely simple “point and click” process. For example, in the “add-on” implementation described previously, the area of interest is defined by drawing a rectangle within a conventional digitised video feed microscope image, and simply pressing “start.” The number of illuminations required to cover the defined area is computed; the diffraction patterns are collected; and the images are processed and displayed (phase; amplitude; and a variety of other application-specific options as desired.)



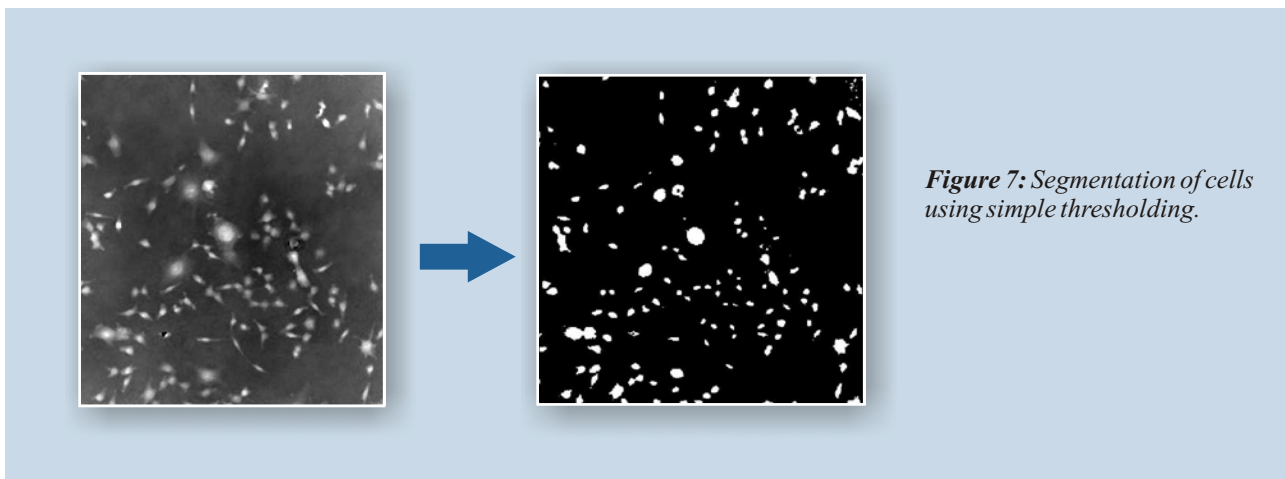
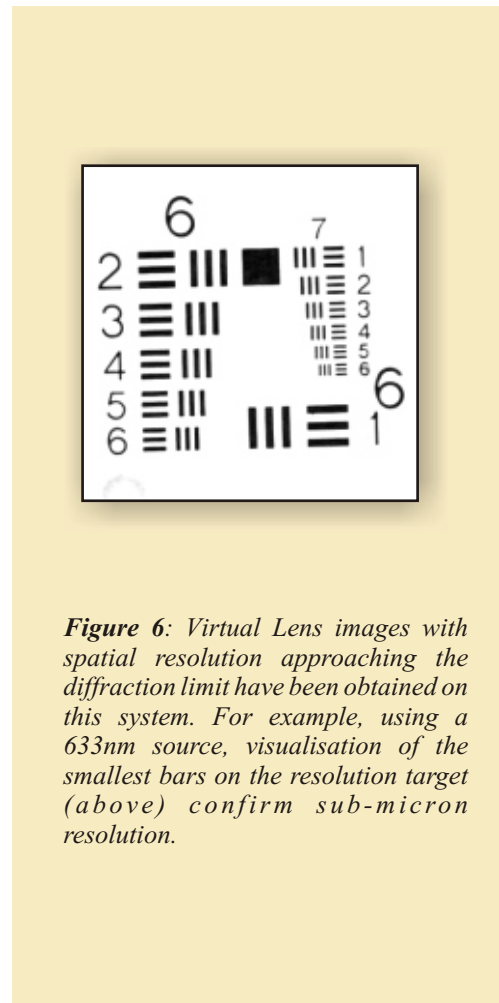
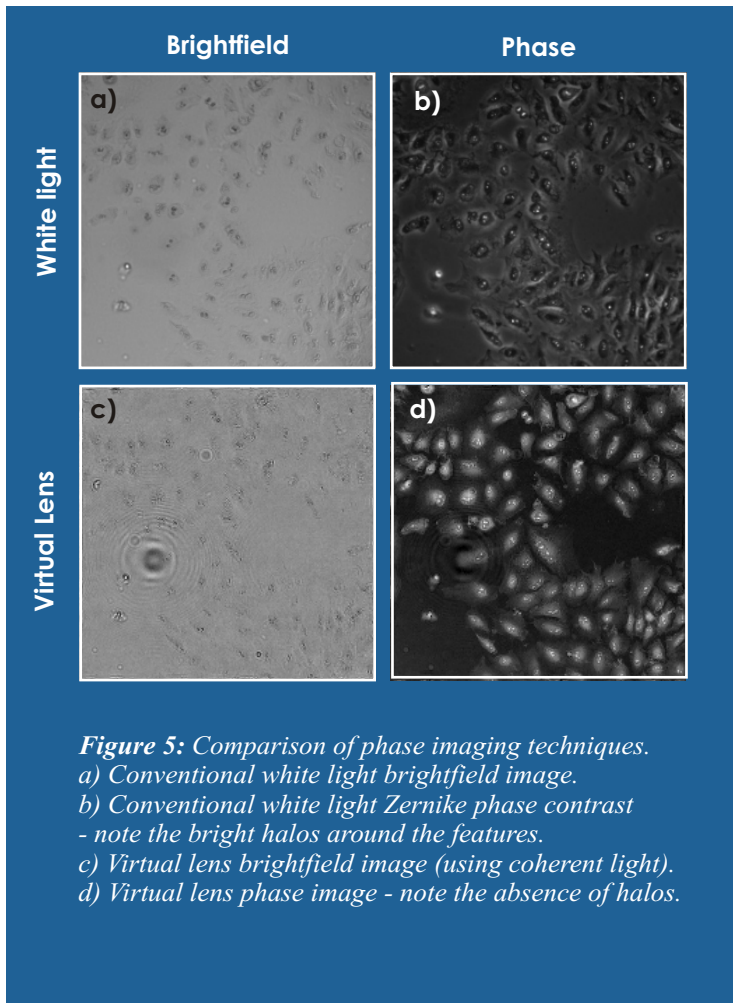
*The user selects, on a real-time video feed conventional microscope image, a region of interest for imaging by the Virtual Lens, then selects "Start Scan."*

*The processed amplitude and phase images (and other optional outputs) are presented for selection, viewing and optional post-processing by the user.*



## II. Virtual Lens image characteristics

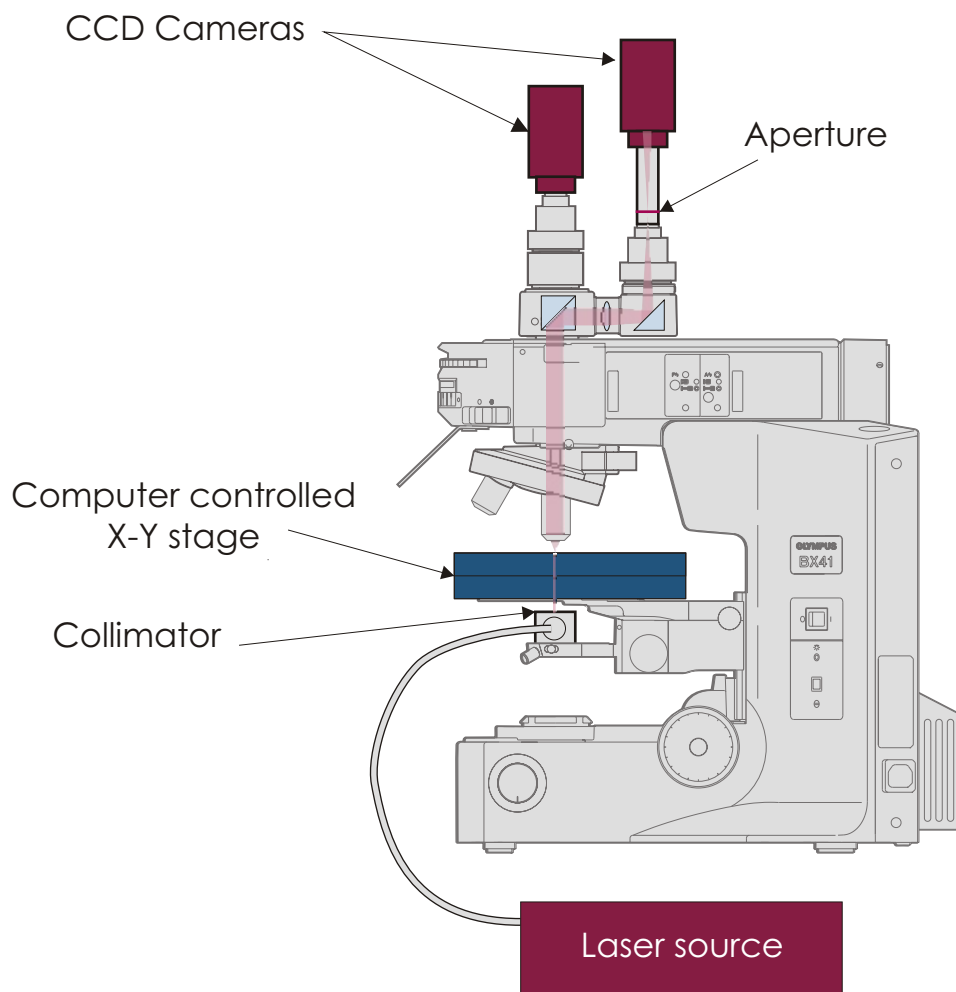
Unstained cells (figure 5) that are essentially transparent in the conventional white light image are rendered visible with very high contrast in the Virtual Lens phase image. Shadow and halo artefacts associated with conventional phase contrast methods are absent. The data lend themselves to simple thresholding prior to segmentation (figure 7.)



## Integration of the Phase Focus Virtual Lens with Existing Instrumentation

The Virtual Lens requires only a coherent light source (e.g.: diode laser); a means for moving the illumination with respect to the specimen; a digital camera (e.g.: CCD); and the Virtual Lens processor itself (the Phase Focus Virtual Lens algorithm installed in an optimised PC/GPU processing environment.) It can be implemented in a "stand-alone" instrument, or can be integrated easily with existing instrumentation. In the latter case, the advantages of the Virtual Lens augment the full complement of existing conventional applications.

By way of example, the Virtual Lens has here been integrated onto a conventional microscope platform (right). The interface components (below) are shown in red (CCD camera; laser source) and blue (motorised stage.) Optionally, Virtual Lens data acquisition protocols may take advantage of existing conventional objective lenses, to integrate or compare the Virtual Lens with conventional techniques. Moreover, in this particular "add-on" configuration, the Virtual Lens may operate in reflected mode as well as in transmission mode.



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UK and international patents pending

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