

Focus on Microscopy: A Technique for Imaging Live Cell Interactions and Mechanisms

by **Barbara Foster**

Since the advent of high numerical aperture (NA) objectives, the practical, accepted limit of resolution for light microscopes has been approximately 0.25 μm (250 nm). A new technology, developed by Dr. Vitaly Vodyanoy of Auburn University (Auburn, AL), produces crisp, high-contrast, and high resolution images, routinely in the 150-nm range (as confirmed with a high-resolution optical test slide; images available at www.CytoViva.com.)

Called CytoViva™, the light microscope accessory (now available through **Aetos Technologies**, Opelika, AL) couples proprietary optics in a customized condenser system with a low-wattage liquid light guide illuminator and a high-NA oil immersion objective with iris (*Figure 1*). The result is a cost-effective, real-time imaging system well suited for observing interactions and definition of mechanisms within live cells and between live cells and small entities such as bacteria, colloids, and viruses.

Resolution versus detection

Most techniques derived from conventional brightfield microscopy (i.e., phase contrast, Hoffman modulation contrast, and differential interference contrast [DIC]) are resolution limited. That is, at some point, features will become so small that the microscope's optics can no longer accurately reproduce size and shape information. At that point, the image information from closely spaced features or neighboring particles merges: Instead of appearing as independent features, the information overlaps and they appear as blobs.

According to Rayleigh's criterion, resolution (R) also depends on a shape factor characteristic of the optics, wavelength of light used, and NA of

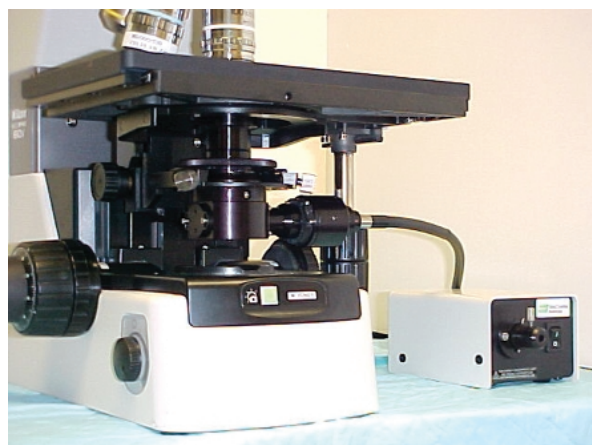


Figure 1 CytoViva consists of a customized condenser and adapter with proprietary optics, a liquid light guide illuminator, and a high-NA objective. (Image courtesy of **Aetos Technologies**.)

the condenser: $R = 1.22\lambda / (\text{NA}_{\text{obj}} + \text{NA}_{\text{cond}})$, where NA is the collecting angle of the objective and condenser. Resolution-limited techniques rely on the ability of the objective to capture light diffracted by the specimen. The combination of the diffracted and undiffracted light forms a diffraction pattern that can be viewed in the back focal plane of the objective (*Figure 2*).

This diffraction pattern carries the code that translates information from the object to the image. The central, zero order (0) carries the background information; any two adjacent orders (e.g., 0, +1) carry the necessary information to accurately reproduce spacing and orientation, and three or more adjacent orders enhance the quality of the edges.

In reality, the diffraction patterns from all the structures in the specimen overlap, generating a homogeneous disk of light in the back focal plane. For clarity, the explanation given here will use the diffraction



Figure 2 Diffraction pattern produced by a simple object and impact of NA. Red ring: small collecting angle of a low-NA objective (e.g., 0.10), white ring: collecting angle of a moderate-NA objective (e.g., 0.44), full aperture: collecting angle of a larger-NA objective (e.g., 1.3 oil immersion).

pattern created from a simple grating made by scribing lines through chrome deposited on glass.

If the objective has a small numerical aperture and only captures the zero order (Figure 2, red ring), the image will only display background; none of the structural information from the real object will be imaged. A larger NA (white ring) collects more of the diffraction pattern, in this case the 0 and +1 or -1 diffracted orders. (Note that capturing -1, 0, and +1 only counts as 2 adjacent orders.) Size and shape will be conveyed from object to image, but the edges will be soft and lack definition. The full NA captures 0, +1, and +2, resulting in images with sharp, clean edges as well as accurate spacing and shape information.

In contrast to these brightfield-based techniques, darkfield is detection limited. Although diffraction occurs, objects far below the limit of resolution can be detected as long as they scatter two or three photons to the eye or camera. Because this technique surpasses the limits of resolution, objects as small as 30 nm can be detected, but no comment can be made about their size, shape, or orientation.

Resolution and detection

While CytoViva's images have a darkfield-like appearance, the optics go well beyond darkfield to com-

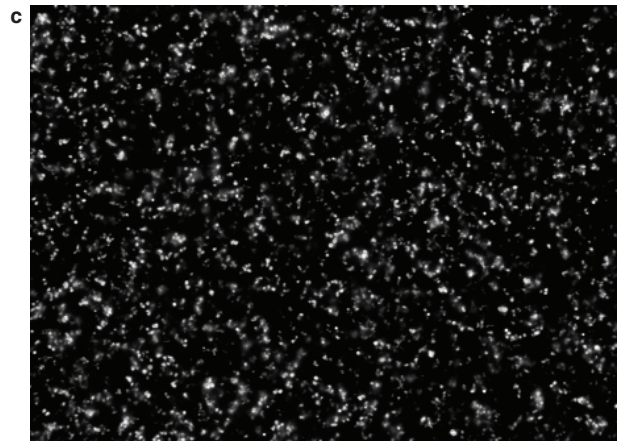
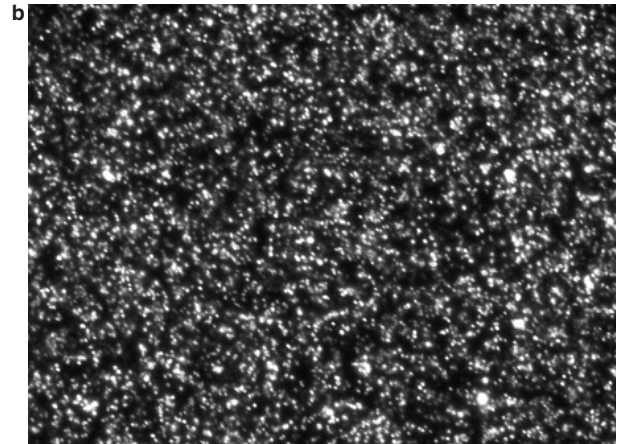
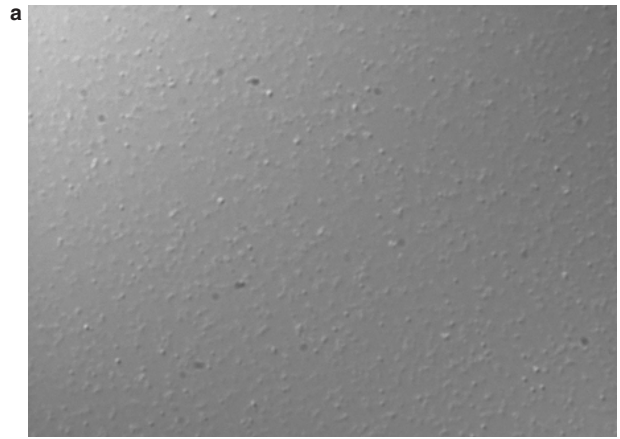


Figure 3 Protein aggregates (100×/1.35 NA oil immersion objective): a) DIC, b) darkfield, c) CytoViva. (All microscopy images courtesy of Dr. Vodyanoy and his research group, School of Veterinary Medicine, Auburn, AL.)

bine resolution with detection. (The exact mechanism is not yet understood but is under study, courtesy of a Fetzer grant.) *Figure 3* shows a comparison between three techniques: DIC (or Nomarski), conventional darkfield, and CytoViva. The features are small (150 nm) protein aggregates. Note that in the DIC image (Figure 3a), the particles are nearly invisible. Because they are so small, the gradients presented to the DIC system exceed the ability of the system to image them.

Darkfield images an infinitely deep depth of field, including information from above and below the plane of focus, creating haze and halo that obscure discrete particle information and distort image size (Figure 3b). In comparison, CytoViva (Figure 3c) images only a shallow plane, producing a crisp, clean background. Additionally, this shallow plane of focus offers the ability to perform optical sectioning, allowing the researcher to investigate cells layer by layer.

Bright images and cool temperatures

Because the technology uses light efficiently, its 24-W light source outshines conventional 100-W halogen illuminators, producing bright images and eliminating the need for expensive and slow low-light level cameras. Use of a liquid light guide to transport illumination from the source also minimizes the thermal impact on living systems.

Ease of use

In the CytoViva system, a patent-pending Ultra Resolution Imaging™ (URI™) condenser system replaces the conventional condenser. The sample is mounted using typical oil immersion procedures, placing a drop of oil between the condenser and the back of the slide, with a second drop on top of the cover slip. The condenser is then raised until a ring of light with dark center appears. The ring is centered to the optic axis using the conventional condenser centration screw.

Tunability

Three components of the system offer opportunities to optimize the image: an iris in the objective, an iris on the light source, and condenser height. Adjustment of the iris on the light source is the simplest of these modifications and balances the intensity of the background to the intensity of the sample.

Condenser height affects both the position of the illumination spot and the energy density. In addition, slight vertical adjustments change the effective depth of observation. Conventional slides range in thickness from 1.1 to 1.3 mm. Adjustment of the condenser height compensates for the varying thicknesses, optimizing the image.

Dr. Vodyanoy's group has also used Lab-Tek™ chamber slides (Nunc/Intermed,

Naperville, IL) for investigating in situ cell culture. These slides have a removable, polystyrene medium chamber with two, four, or eight wells. The medium chamber is mounted on a microscope slide that allows the user to inoculate, incubate, fix, stain, and examine in one complete system. The Auburn group has observed and recorded live cells in these chambers with samples as thick as 300 μm, significantly expanding the range of usable sample preparations from regular slides to growth chambers.

Altering the iris in the objective has two effects. First, as seen by removing an eyepiece and peering into the back focal plane of the objective, the iris can be closed to match the size of a modulating ring mounted in the condenser, resulting in richer black backgrounds. As with most microscopy techniques, opening the condenser maximizes numerical aperture and therefore resolution. Smaller particles, not visible when the iris is closed, can be resolved when the iris is opened.

New vistas and new mechanisms

CytoViva has already had an impact on how biologists explain interactions with and between cells. Several examples are discussed below.

Spirochaete infestation

Spirochaetes are a family of bacteria responsible for a number of human ills ranging from Lyme disease to sexually transmitted diseases. The conventional explanation for spirochaete infestation is derived

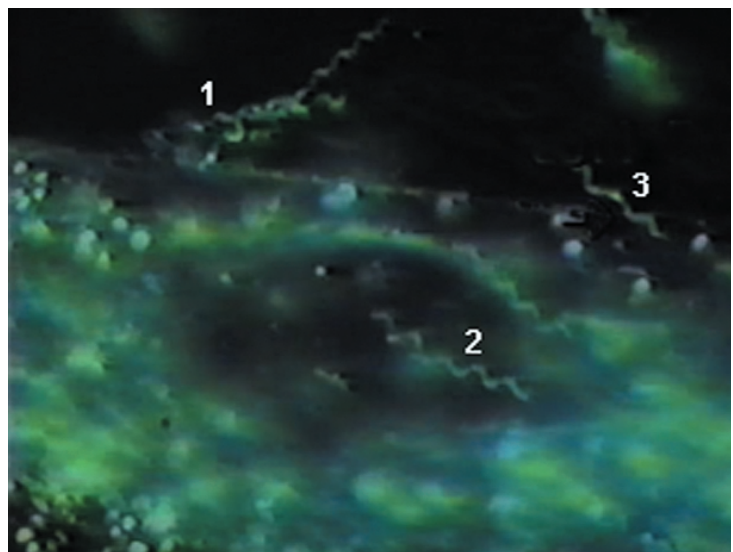


Figure 4 CytoViva presents new explanations for spirochaete infestation. Spirochaetes (1) conjugating (2) within a cell and (3) exiting the cell (100×/1.35 NA oil immersion objective).

from static transmission electron microscopy (TEM) and fluorescence images. According to this mechanism, the spirochaetes lie on the surface of a cell and are engulfed by the cell. Research using the CytoViva system¹ (Figure 4) suggests a very different mechanism. Movies from these studies (available at www.CytoViva.com) reveal that the spirochaete actually bumps along the surface of the cell “nose” first until it finds a susceptible location, attaches, then stays dormant until some yet-to-be-determined signal activates it. At that point, it energetically screws itself into the cell; infects it; and then, interestingly, exits the cell along the same path.

Neutrophils

Because of its ability to minimize information-obscuring scatter, CytoViva has been used to analyze how neutrophils (a type of white blood cell) interact with damaged red blood cells (erythrocytes). It is well known that macrophages internalize and digest damaged erythrocytes, but neutrophil-erythrocyte interactions have not been widely reported. CytoViva images reveal that some erythrocytes actually dock with neutrophils. The neutrophil then repairs and cleans the membrane, releasing the healed erythrocytes when finished.

Apoptosis

The mechanism of cell death is critical to understanding how to save healthy cells and how to destroy unwanted cells such as cancer. Using CytoViva, researchers in Dr. Vodyanoy's laboratory have elucidated several mechanisms for cell death. Figure 5 shows one example: a brain cancer cell called glioma. In the earliest stages of dying, the cell expands dramatically and forms blisters or blebs (Figure 5a). In the final stages, the delicate cell membrane breaks, releasing the cell contents (Figure 5b). CytoViva offers two distinct advantages for observing and recording this phenomenon: It enhances contrast and adds the ability to optically section so that the researcher can zoom in on a specific plane of interest.

Brave new world?

CytoViva is not for everyone. However, for those researchers struggling to define processes within and

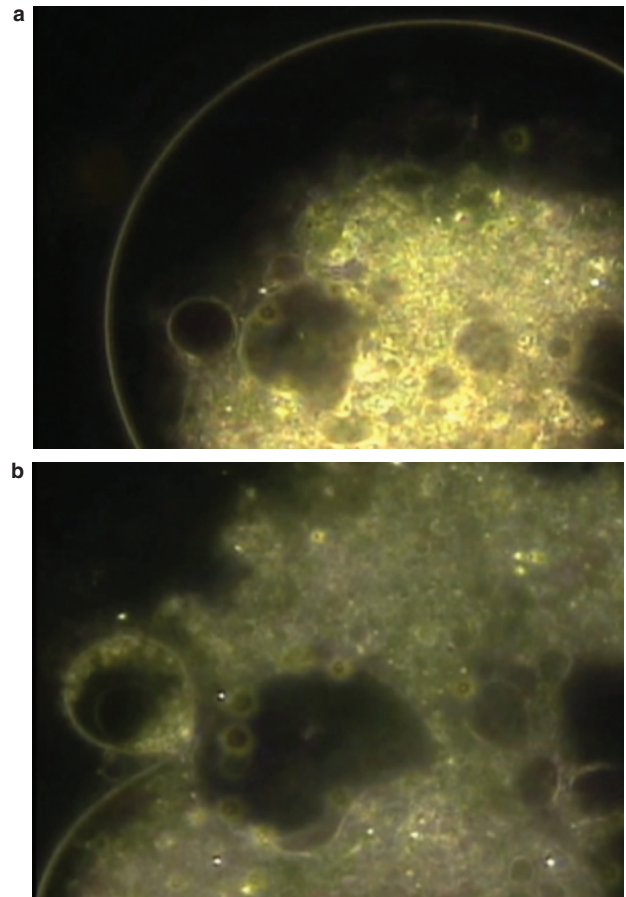


Figure 5 Glioma (brain cancer) cell (a) expanding and forming blebs in its early stages of dying and (b) expelling its contents in its final stages (100×/1.35 NA oil immersion objective).

between living cells, especially those processes involving very small entities, it quickly and easily offers real-time information and solutions.

Reference

1. Chambers M, Reddy G, Vodyanoy V. Attachment of *B. burgdorferi* to cultured mammalian cells. Abstracts of the 1995 American Association for the Advancement of Science, Feb 1995, Atlanta, GA:140.

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