

# Media Cybernetics White Paper

## Extended Depth of Field

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### Introduction

The narrow depth of field of the light microscope is either an asset or a liability depending on the needs of the microscopist. Occasionally, by limiting the image to a narrow plane in the vertical axis, it helps focus the observer’s attention by isolating structures in a thick sample, permitting the viewer to concentrate on the pertinent object while ignoring distracting elements above and below the plane of focus. However, in many if not most cases, this feature is a liability when the object’s importance lies in its three-dimensional configuration and portions of it are rendered invisible when they fall outside the depth of field of the objective lens. In this case, critical structures are lost from view when an image is displayed for publication.

### Numerical Aperture and Depth of Field

For most histological specimens the depth of field is much smaller than the thickness of the specimen. Below is a table that shows the depth of field as a function of an objective lens’s numerical aperture.

Numerical Aperture	.25	.30	.5	.65	.85	.95
Depth in $\mu\text{m}$	8.00	5.50	2.00	1.00	.25	.10

Table 1- Depth of Field as a Function of Numerical Aperture. As the numerical aperture increases, the depth of field decreases. Thus, in order to obtain higher lateral resolution a microscopist sacrifices information from the vertical axis. The values assume that the investigator is using green light. Table adapted from Kodak’s publication, Photography through the Microscope.

### Extending the Depth of Field

To overcome this limitation, an experienced microscopist, when viewing through the microscope, continually adjusts focus, moving the zone of sharpness up and down the optical axis to form a mental image of a structure’s three-dimensional shape. Unfortunately, this strategy cannot be duplicated in a static paper publication and its application to web sites or platform preparations can be difficult. Alternate strategies must be employed to show the object’s overall shape.

One method, available to any microscopist who captures images with either film or electronic medium, is to use a smaller numerical aperture. This can be done most conveniently by simply closing down the condenser iris diaphragm. Using this strategy there is a significant increase in the depth of field of a single captured image. For example, in Table 1, it can be seen that by reducing the effective numerical aperture from 0.85 to 0.65 the depth of increases from .25  $\mu\text{m}$  to 1  $\mu\text{m}$ . Unfortunately, this procedure will result in a loss of resolution and the loss of definition can limit or destroy the image’s utility. If we look at Equation 1, for calculating the theoretical resolving power as a function of a lens’ N.A., where R is the resolving power in  $\mu\text{m}$ ,  $\lambda$  is the wavelength of light in  $\mu\text{m}$  and N.A. is numerical aperture, we can see that a .85 lens, when using green light .55  $\mu\text{m}$ , will have a resolving power of 0.32  $\mu\text{m}$ , while a .65 lens will have a resolving power of 0.42  $\mu\text{m}$ .

$$R = \frac{\lambda}{2N.A.}$$

Equation 1. R is the resolving power in  $\mu\text{m}$ ,  $\lambda$  is in  $\mu\text{m}$ , and the resolving power of a lens with a numerical aperture of 1.4 is approximately 196 nm for light of a wavelength of 550 nm.

Another approach for displaying three-dimensional objects in two-dimensional format is by either drawing or projection. Neuroscientists are familiar with the work of Ramón y Cajal, who laboriously sketched images of neurons at different focal planes and combined them onto a drawing that dramatically illustrated their spatial distribution. Historically, this technique was the superior method for illustrating the branching pattern of axons and dendrites and it surpassed photographic techniques for illustrating a cell's three-dimensional features in a plate.

Fortunately, if the user has access to an electronic imaging camera and the appropriate software, the pioneering work of Cajal can be duplicated with much less effort, at a considerable gain in accuracy and speed. Simply, this technique known as either extended depth of field or projection requires capturing several images of a specimen at different heights. By using a high numerical aperture lens, the microscopist can gather images at higher resolution than that obtainable by using a decreased numerical aperture. These captured images are merged into a composite micrograph that shows the various features that would normally lie outside a single plane of focus. This strategy, which is familiar to users of the confocal microscope, can be applied to brightfield images and is called extended depth of field. For the purposes of this paper, the authors used the image processing software, Image-Pro Plus, from Media Cybernetics.

To illustrate the efficacy of this procedure we collected images of blood cells within the lumen of a human kidney vessel. Figure 1 was captured within the center of the section and many of the red blood cells are blurred by lying outside the plane of focus. In addition, the nuclei of three white blood cells are seen as indistinct dark shadows since they lie outside the plane of focus.

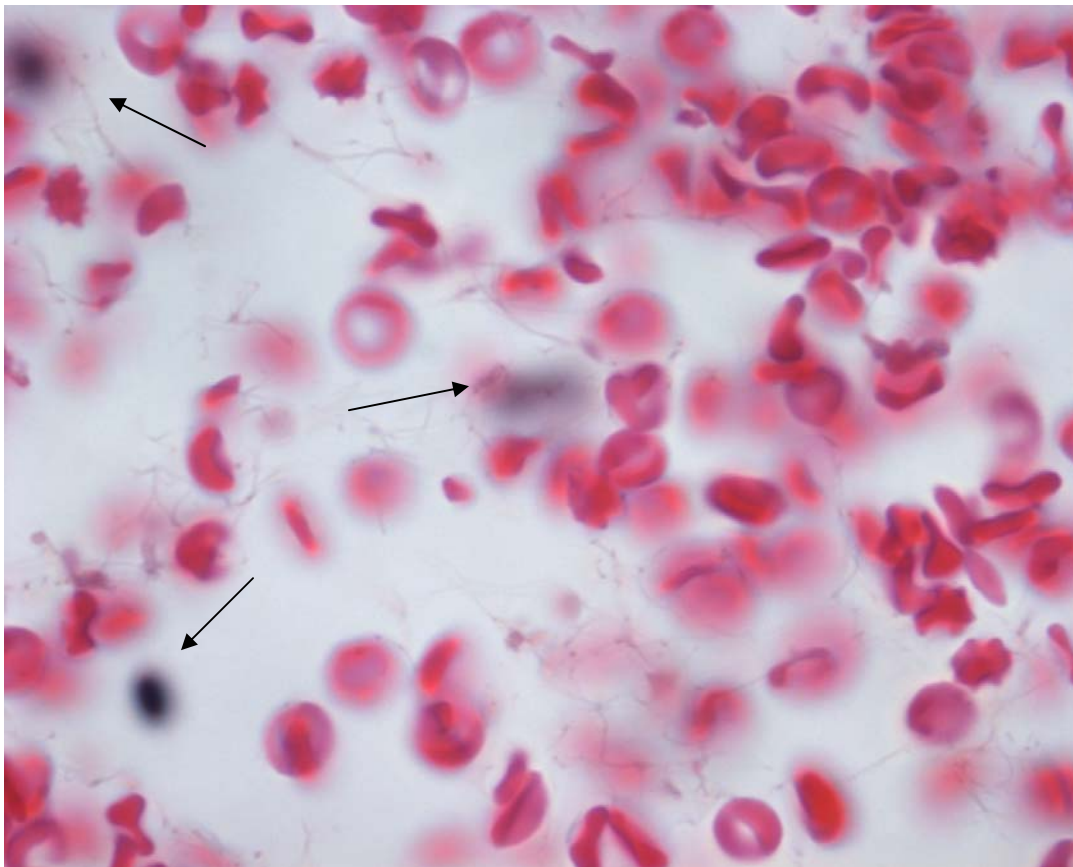


Figure 1. Out of focus white blood cells indicated by dark arrows.

By harvesting 15 sections, at different planes within the section, one can achieve the micrograph below by using an extended depth of focus function. In the micrograph below, the majority of red blood cells now are rendered with sharp definition. More importantly, one can identify three white blood cells that were invisible in the single plane image.

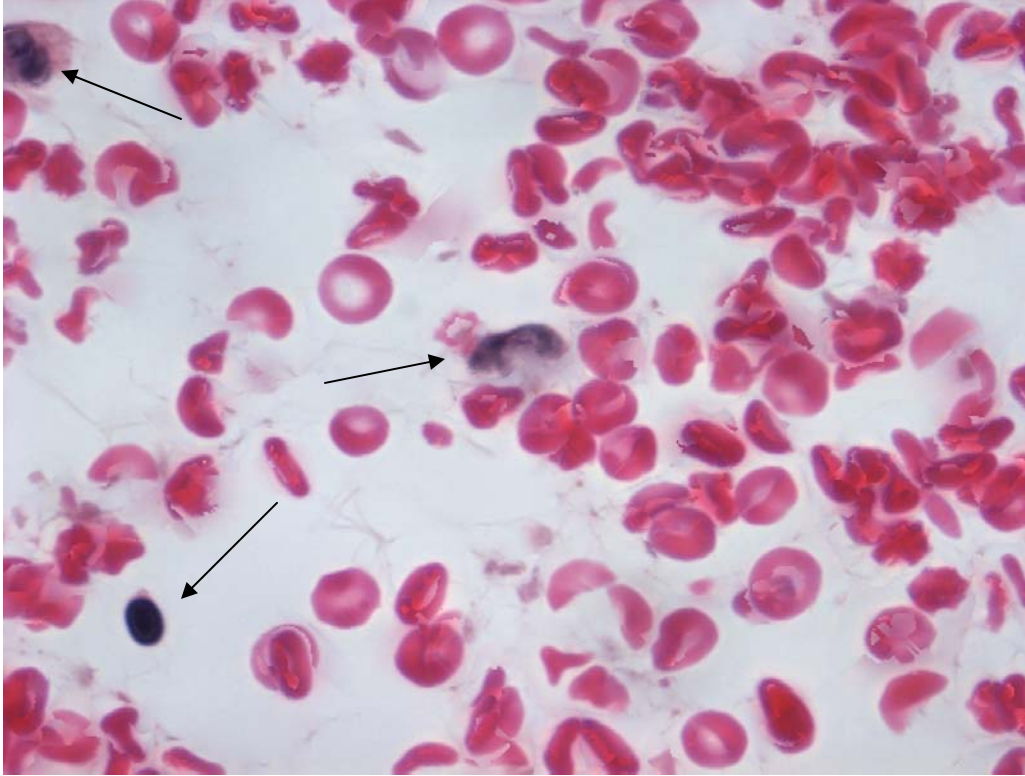


Figure 2. Extended depth of field rendering sharp focus to both red and white (indicated by dark arrows) blood cells.

### An Example Using Neurons

The inclusion of detail within the micrograph is rendered more obvious in the study of silver impregnated neurons. In the neurosciences, the investigator Ramón Cajal is recognized for his exquisite drawings that showed the distribution of dendritic arborization and the path of axons in brain tissue. By meticulously drawing the image of a neuron at different planes of focus, Cajal overlaid the images to provide a projection of the three-dimensional network of nerves in two dimensions. A task that was accomplished before the development of digital cameras and electronic computers and whose implications to neuron function led to his winning the Nobel Prize in Physiology and Medicine.

This type of work can be implemented with digital images and by electronic projection. Although the neuron is opaque and the specimen is monochrome, extended depth of field works effectively with this traditional neuronal type of specimen. The speed with which images can be acquired and projected can facilitate the analysis and interpretation of the specimen. Below is an image of a neuron at the plane of its cell body. The dendrites can be seen to passing through the region of focus and becoming blurred as they ascend or descend through the section (Figure 3).

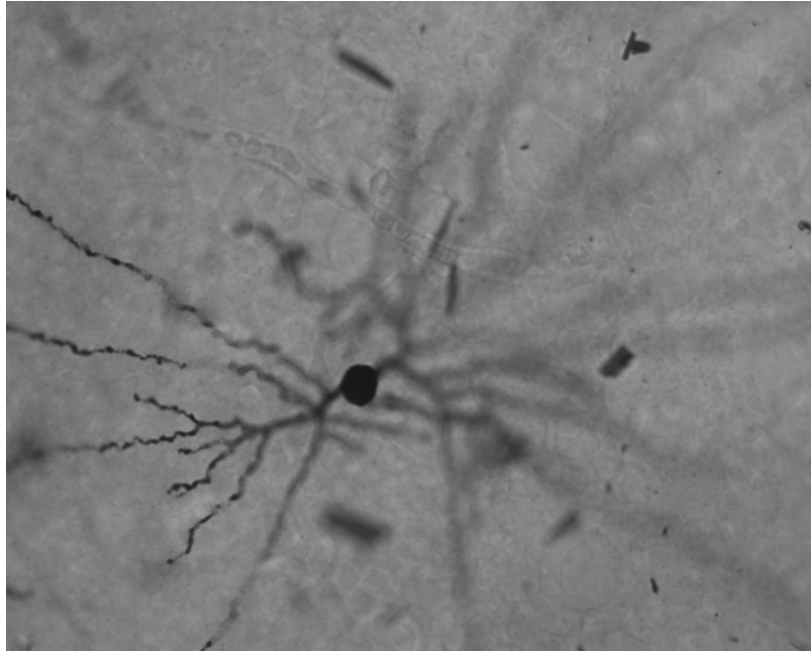


Figure 3. Dendrites descending through optical sections.

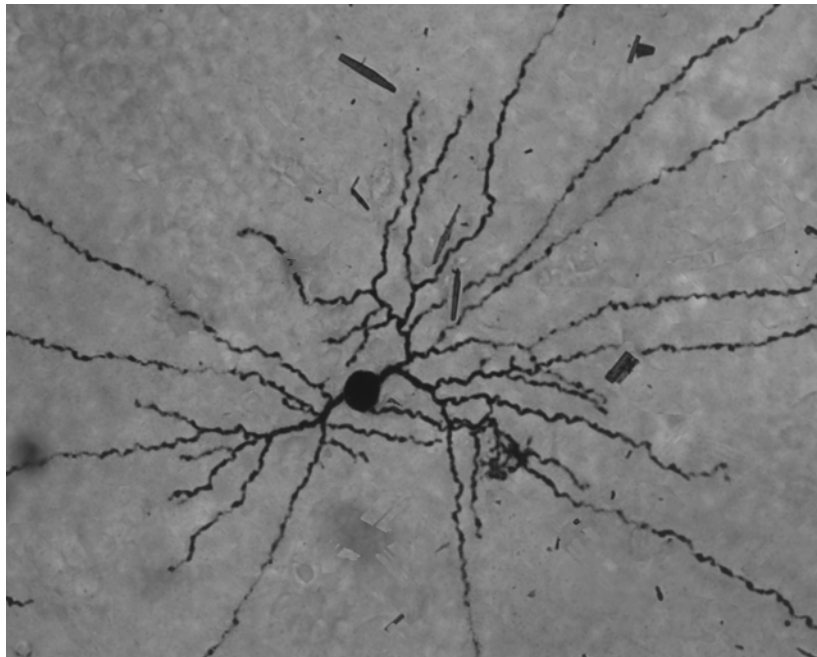


Figure 4. Extending the depth of field to view dendrites.

By projecting the stacked images of the neuron at different planes, one has an extended depth of field, better displaying the extent of neuronal processes (Figure 4).

#### **Extended Depth of Field and Nomarski DIC**

Extending the depth of field is applicable with modern microscopy techniques, such as Nomarski Differential Interference Contrast. With this contrast enhancing modality, the microscope generates excellent optical sections, facilitating the harvesting of precise optical planes. Below are two pictures of a human squamous epithelial cell, one showing the surface membrane ridges and an out of focus nucleus, and the other showing a cleanly focused nucleus (Figure 5).

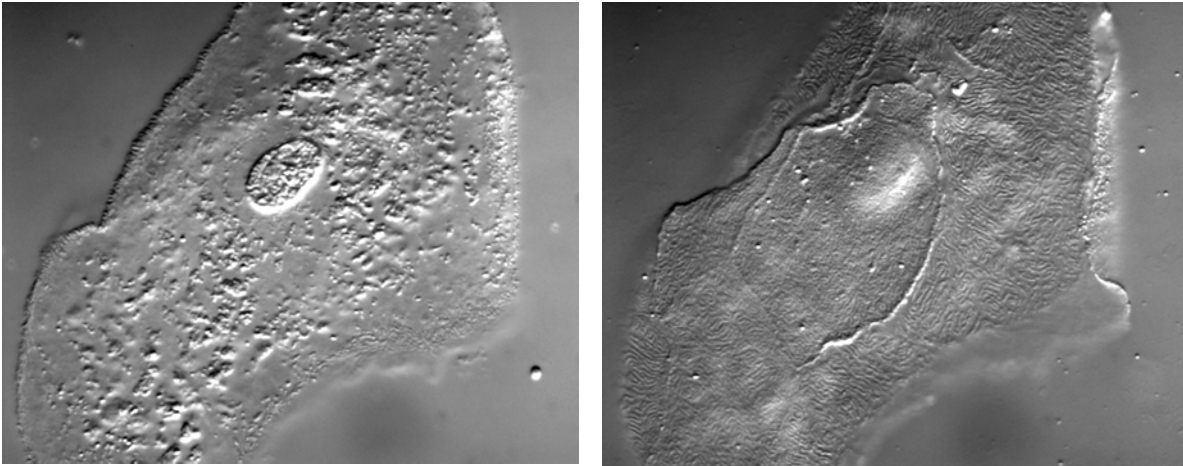


Figure 5. Human squamous epithelial cell viewed under Nomarski Differential Interference Contrast. Different optical sections are shown revealing the nucleus (left) and surface membrane ridges (right). If one sections to the center of the cell, the out-of-focus nucleus is brought into sharp registry. However, the narrow depth of field obscures all the epithelial ridges.

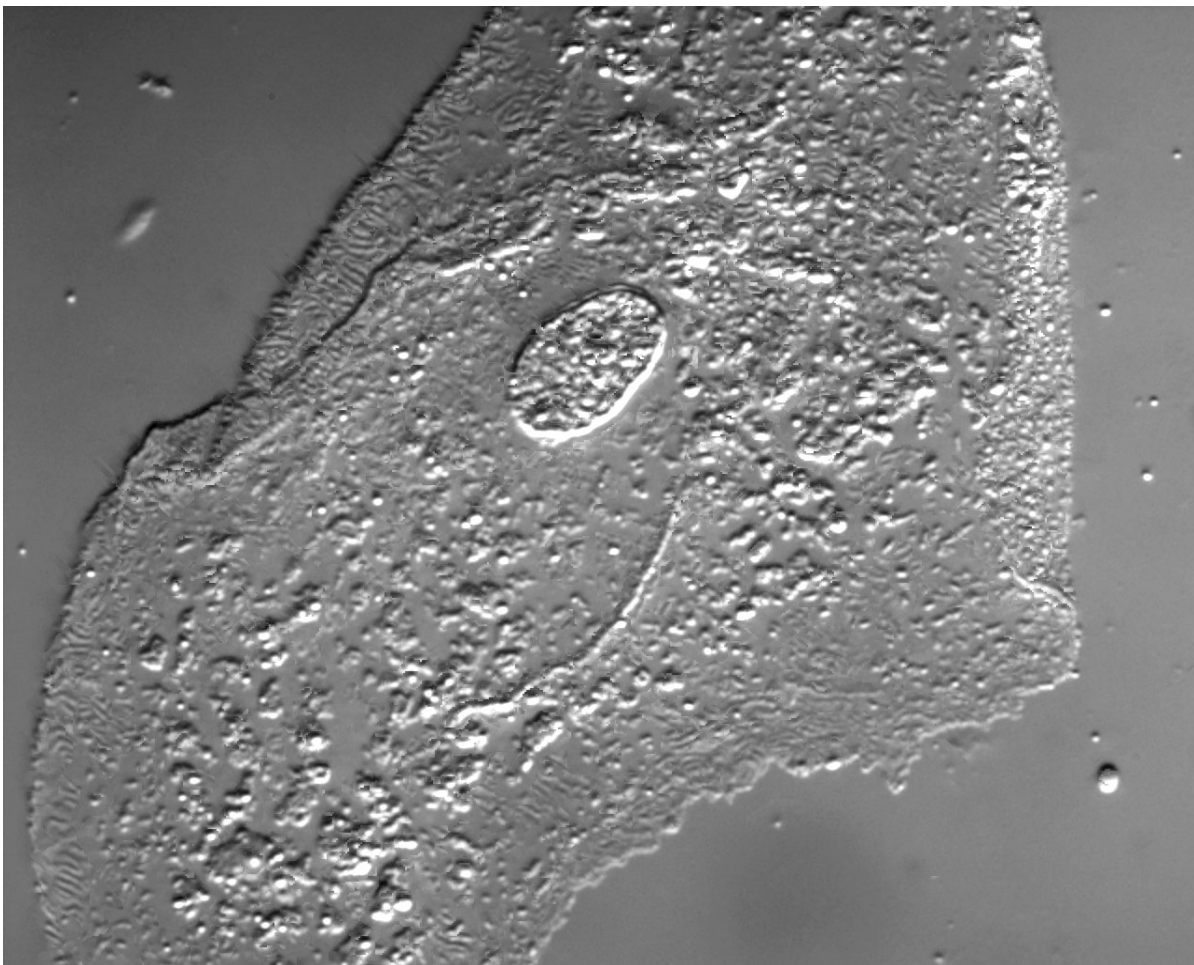


Figure 6. Extended depth of field showing membrane ridges and nucleus in sharp focus.

By taking a stack of sections, it is possible to display the epithelial cell's nucleus and its membranous ridges in a single micrograph (Figure 6).

In conclusion, the extension of a microscope's depth of field can be achieved with stained histological samples, simple monochromatic staining procedures such as silver impregnation, or with modern contrast enhancing techniques such as Nomarski Differential Interference Contrast. In those image series where the specimen's structure cleanly disappears from focus, it is possible to generate a more realistic display of cellular and histological structure than obtainable in a single plane of view. Such a methodology provides a cost-effective and convenient means of illustrating publications.

## References

Delly, J.G. Photography Through the Microscope. 1980. Eastman Kodak Company. Rochester, NY.

Inoue, S., and Spring, K. R. 1997. Video Microscopy: The Fundamentals (2<sup>nd</sup> Edition). p. 27-32. Plenum Press, New York, NY.

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