Ultra-high Contrast Amplification in Bright-field Images

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Introduction

In this paper, a new way for visualization of unstained transparent specimens is described, which is based on bright-field imaging and promises an improved resolution and contrast. The final results can be compared with conventional phase contrast images, and the new method may lead to superior results in most specimens.

In standard bright-field illumination, colorless specimens such as unstained cells and microorganisms (so-called phase objects) are just barely visible because of their low optical density. These specimens do not absorb light in a relevant manner; they only modify the phase of transmitted light by about one-quarter wavelength (λ/4). Such small differences in phase are associated with minimal differences in density, and they cannot be perceived directly by the eye or photographic film. Thus, phase contrast is most widely used for examinations of unstained specimens. The principles of phase contrast and all optical components necessary for this, have been described by its inventor, the Dutch physicist Frits Zernike [1–2].

The optical pathway of phase contrast is shown in Figure 1 (modified from [3]). To achieve phase contrast, two components of bright-field microscopes must be modified: (a) The condenser has to be equipped with a ring-shaped aperture or mask (the condenser annulus), which is placed near the condenser aperture diaphragm. (b) A conjugate phase plate (or ring) is placed in the back focal plane of the objective. The condenser annulus and the phase ring in the objective have to be optically aligned so that they are conjugate. With this arrangement, the specimen is illuminated by the apex of a cone of light. The light beams, which are diffracted by the specimen, pass through the objective lens at various angles that are dependent on the relative refractive index, thickness, and superficial texture of the specimen. The other light components, corresponding to the background, pass through the phase ring in the objective, which produces an additional phase difference. Thus, the phase differences between the specimen, its details, and the background are amplified in the final image, so that minimal differences in refractive index are visible even in thin colorless specimens with low contrast.

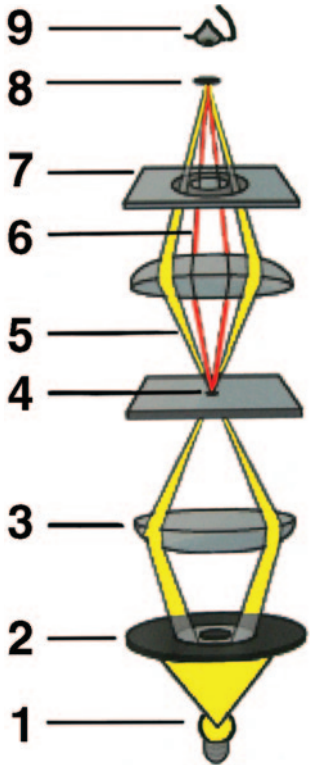

Figure 1: Simplified optical pathway for phase contrast microscopy (modified from [3]). Key: 1 = light source, 2 = annular shaped light mask, 3 = condenser; 4 = stage/specimen, 5 = background light (yellow), 6 = light bent by the specimen (red), 7 = phase ring/phase plate, 8 = eyepiece with intermediate image, 9 = eye.

Depending on the configuration and properties of the phase ring in the objective, the natural phase shift within phase preparations (circa λ/4) is amplified, so that the resulting final difference in phase of the specimen and the background is around one-half wavelength (λ/2, positive phase contrast) or one wavelength (λ, negative phase contrast). In positive phase contrast, the specimen is visible with medium or dark grey features, surrounded by a bright halo, and the background is of higher intensity than the specimen. In negative phase contrast, the contrast of these features is inverted. In both methods, the intensity of contrast is solely determined by the design of the objective lens phase ring and the optical density of the specimen and its surrounding medium. Because phase contrast is usually optimized for observations of native cells in their natural environment and calculated for an amplification of λ/4-phase-shifts, the quality of traditional phase contrast images will be degraded the more the natural phase difference deviates from λ/4.

The advantages and limitations of modern phase contrast microscopy have been compiled by several authors [4–6]. Standard phase contrast is affected by several specific limitations: (a) Halo artifacts are prevalent, especially in specimens, which induce large phase shifts. (b) The condenser iris diaphragms should always be wide open so that the contour sharpness and the depth and planarity of field cannot be influenced by the condenser aperture. (c) The intensity of contrast cannot be adjusted with regard to the optical characteristics of the specimen, and all existing phase shifts remain constant and cannot be trimmed. (d) The phase ring within phase contrast lenses may reduce the image quality in general when compared with corresponding lenses designed for bright-field. (e) Phase contrast images may be seriously degraded in certain circumstances. For instance, the character of phase contrast images can be modified into low-quality, low-contrast bright-field images when living cells must be examined that are cultured in small volumes [7].

To overcome these difficulties, digital bright-field images may be taken from phase objects and rendered through a new multi-step procedure such that small differences in density corresponding to small phase differences can be successively amplified. This technique, called ultra-high contrast amplification, leads to images showing more detail and fewer artifacts than corresponding images taken in standard phase contrast.

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Suitable software solutions and the first practical results of this method are described in this paper.

Principles of Ultra-high Contrast Amplification

The term dynamic range is defined as the ratio of the maximum to the minimum values of luminance within a specimen or image. In general, only a small part of the existing dynamic range in the respective natural environment can be detected by normal technical means (Table 1). Images with dynamic range up to 10,000:1 are defined as low dynamic range (LDR) images. In high dynamic range (HDR) images, for example, normal daylight scenes, the visible dynamic range is much higher, in most cases 100,000:1 to 1,000,000:1.

In unstained phase objects, the natural dynamic range is much lower than in any other kind of specimen. Thus, existing low differences in density associated with minimal differences in regional brightness and contrast must be amplified by high-dynamic-range rendering (HDR rendering) as a first step. The method of HDR rendering has been developed, evaluated, and described by several authors [8–13]. In principle, software for HDR rendering can modify pre-existing original images in two ways:

1. Contrast and brightness equalization. When specimens or illumination modes are associated with a large variation in object brightness and a high range of contrast, the specimen has to be photographed at different exposures, and the respective image stack has to be superimposed and rendered by the respective HDR-rendering software. As some parts of the specimen appear over- or under-exposed in all original single images, some details within the specimen cannot be well recognized in these images because they are too much darkened or brightened. Differences in luminance greater than around 1 to 2 EV can lead to degradations of the visible visual information in one-shot photographs. As a result from HDR rendering, all pre-existing ultra-high ranges in contrast and brightness are equalized, and the final image reconstruction is free from any visible over- and under-exposed zones so that existing details within the specimens can be documented in an optimized clarity. Any degradation of image quality and visual information caused by over- or under-exposure is eliminated. Moreover, in some software solutions, pre-existing differences in brightness and contrast can also be transformed into a well-balanced multi-color contrast, which is also characterized by a high constancy in brightness.

2. Contrast amplification. When specimens or illumination modes are affected with a very low variation in brightness and contrast (for example, bright-field images of unstained phase specimens), the same software solutions can act as ultra-high contrast amplifiers. Also in this case, two or more single images have to be superimposed; otherwise, HDR-rendering cannot work. When the respective low-contrast images are superimposed with each other and HDR rendering is appropriately carried out, all pre-existing low-contrasted details, which may just barely be visible in the original images, can be transformed into a high-contrast appearance. It is important for the authenticity of the visual information within the final reconstructed images that only pre-existing details that are situated in at least one of the original images and associated with pre-existing low differences in brightness be amplified and high-contrasted by HDR rendering. From practical evaluations carried out so far, it does not appear that HDR rendering produces any new visible artifacts. Only pre-existing artifacts that may already exist in the original images can be amplified by the rendering procedure. In other words, all information that is amplified by HDR image processing comes from the original images.

Ultra-High Contrast Amplification. To obtain HDR images, the color channels, corresponding to red, green, and blue, and the alpha channel corresponding to the transparency, are transformed from 9 to 32 bit. Moreover, all tonal values are coded as floating point data. In this way, the number of tonal values can be enlarged from 256 (typical of 8-bit images) up to around 4.3 billion graduations per channel. Such high graduations can neither be printed nor observed on a standard screen in a satisfying manner. When HDR images have to be directly visualized, a special screen (HDR monitor) is necessary. Therefore, HDR images must be transformed into new LDR images when they are to be visualized on a normal screen or printed by a normal printer; this separate step in image processing is called tone mapping. When tone mapping is adequately carried out, the resulting final image shows more tonal nuances, sharpness, and detail in fine structures; it also is free from visible over- or under-exposed zones. Also very low natural differences in brightness, corresponding to minimal differences in density and very discrete local phase shifts, are transformed into high contrast. Figure 2 illustrates the HDR rendering of a bright-field image from an unstained thin-layer crystallization. Before the tone mapping procedure is carried out, the HDR image is "overridden" and shows a severe color shift (right part of the screen). After tone mapping (left part of the screen), the final image is well balanced with regard to brightness, sharpness, contrast, and coloration. It appears like an optimally contrasted phase contrast image.

When the procedure of tone mapping is finished, the resulting reconstructed phase image can be optimized further in additional steps with the help of normal digital image processing. In particular, the gradation, histogram, brightness, and contrast level can be re-adjusted in tiny steps so that the contrast can be optimized with regard to the existing real density and phase shift (digital contrast trimming). Each unstained phase structure is documented in optimum contrast, and the regional contrast is no longer determined by the optical density within the specimen and the

Table 1: Typical values of dynamic range for various imaging methods compared with the human eye.

	Low Dynamic Range	High Dynamic Range
Photo prints	1:2 – 1:64	
Screens (laptop, TV)	1:100 – 1:500	
Digital camera	1:1000	
Analog (film) camera	1:10,000	
HDR imaging		1:10,000 – 1:100,000
Human eye		1:100,000 – 1:1,000,000

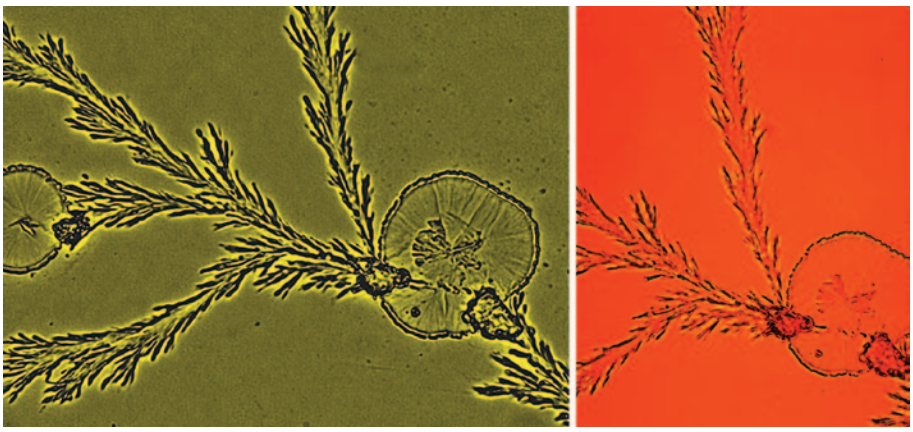

Figure 2: Main screen in Photomatix Pro. Right window: part of the genuine "overrided" HDR image. Left window: preview image showing the results of tone mapping.

surrounding medium. Digitally reconstructed color images taken from colorless specimens should be transformed into black and white.

As alternative solutions for HDR imaging, specialized HDR cameras have recently been developed in order to obtain HDR images in a direct way. The respective algorithms necessary for HDR imaging are integrated within these cameras so that all images are taken according to the HDR standard. The respective HDR images can only be viewed on the special HDR monitors mentioned above.

Materials and Methods

All images were taken with a 7.1 megapixel digital camera (Olympus Camedia C-7070) using a Leica laboratory microscope equipped with a Leica Vario-Photo-Ocular 5-12.5× and a ZERNIKE Universal condenser for bright-field and phase contrast. Objectives for phase contrast and bright-field (the latter without a phase ring) were mounted as doublets so that identical views of unstained phase objects could be taken in conventional phase contrast and bright-field, using phase contrast and bright-field lenses.

Several software solutions are available for DHR rendering (conversion of a normal LDR image into an HDR image) [8–13]. According to our own experience, the software Photomatix Pro (available from the web [14]) leads to the best results in practice when contrast amplification has to be carried out on bright-field photomicrographs. Thus, all contrast-amplified bright-field images presented here were rendered with Photomatix Pro. Photoshop or Photoimpact were used for final post-processing (adjustments of gradation, histogram, brightness and contrast, and black-and-white imaging). Photomatix Pro needs at least two separate single images, which have to be superimposed for HDR rendering. Thus, either two bright-field images were taken from a specimen in identical views, or only a single image was made in bright-field and afterwards duplicated on the computer by renaming and resaving. Photomatix Pro offers several different tools and presets for image rendering. For ultra-high contrast amplification of bright-field images of unstained phase objects, tone mapping was carried out using the "Details Enhancer." Using this tool, several parameters can be manually influenced by the user: strength of contrast enhancements, color saturation, light smoothing, luminosity, white and black point, gamma, color temperature, saturation of highlights and shadows, micro-contrast, micro-smoothing, contrast enhancements in highlights and shadows, and shadow clipping. To obtain suitable quality in this special task, the strength of contrast was set to the maximum level ("100") when low regional differences in brightness had to be amplified. Moreover, the light smoothing had to be regulated to a high level. In unstained phase objects, the color saturation had to be reduced to a low level to achieve a good reproduction of all tonal values. When color saturation was set to "zero," phase structures were directly amplified in black and white. More extensive descriptions of and recommendations for the appropriate use of HDR software in photomicrography have already been published elsewhere [15, 16].

Results

Figures 3–5 demonstrate that digitally reconstructed bright-field-based images from unstained specimens (phase

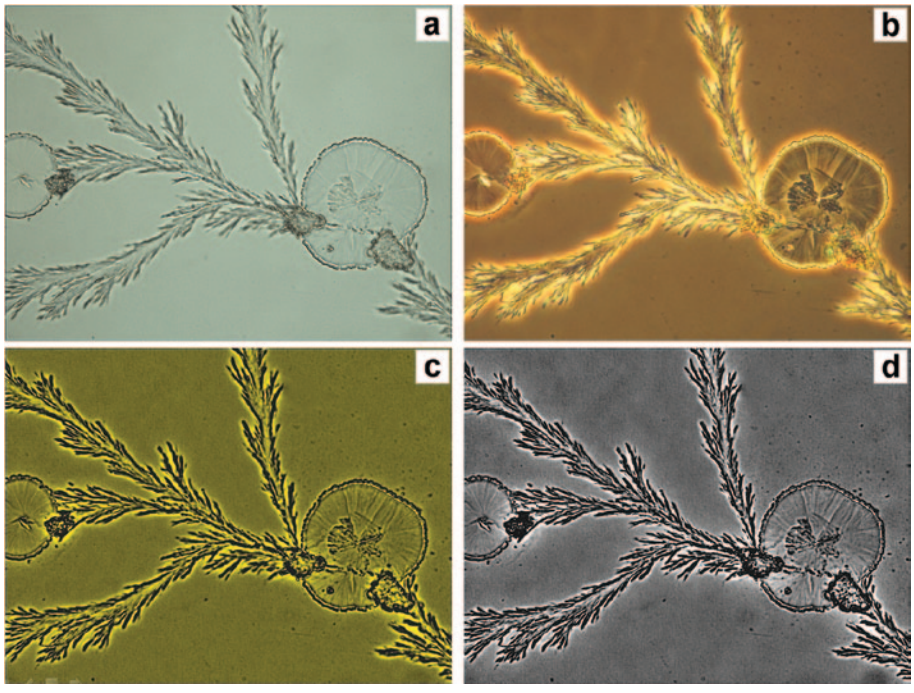

Figure 3: Unstained thin-layer crystallization (specimen from Figure 2), conventional bright-field (a), normal phase contrast (b), contrast amplified bright-field (c), black and white variant (d). Objective 40×, ocular 10×, image width = 100 µm.

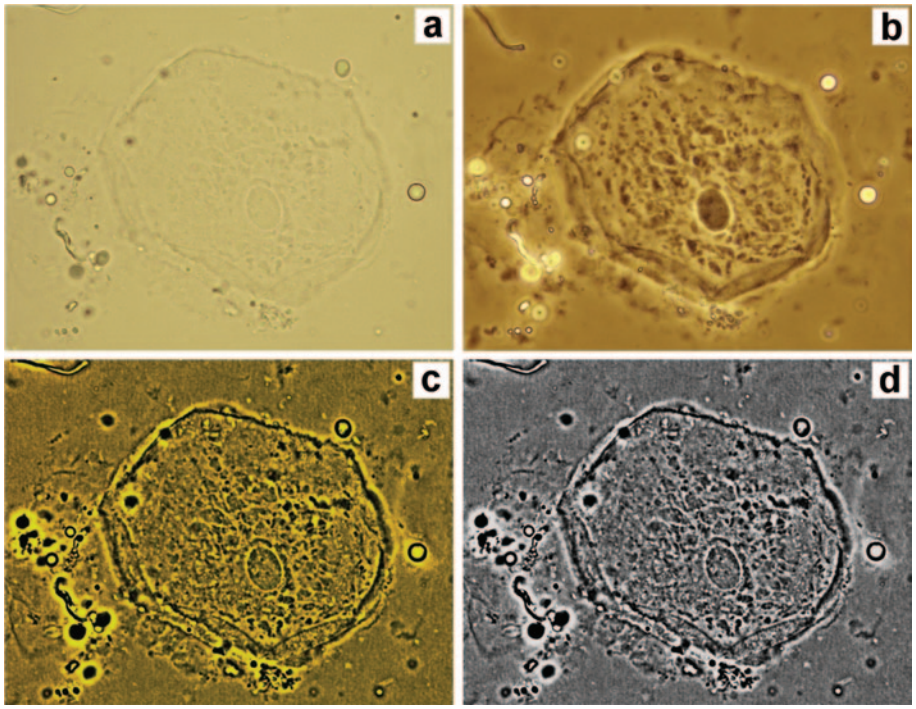

Figure 4: Native epithelial cell from the oral mucosa, conventional bright-field (a), phase contrast (b), contrast amplified bright-field (c), black and white variant (d). Objective 40×, ocular 10×, image width = 70 µm.

objects) can lead to improved contrast and detail, optimized sharpness, and enhanced depth and planarity of field. Figure 3 shows the thin-layer crystallization taken in (a) normal bright-field, (b) common phase contrast, (c) reconstructed by HDR rendering, and (d) HDR rendering converted to black and white. The phase contrast image contains haloing and blooming, whereas the clarity of all structures is improved when HDR rendering is carried out. In the original bright-field image, the colorless thin crystals appear with the lowest clarity and contrast.

Figure 4 demonstrates the usefulness of the new method with regard to observations of native and unstained cells. The epithelial cell of the oral mucosa is just barely visible in (a) bright-field because of its low contrast. In normal phase contrast (b) the cellular structures appear in higher contrast, which is usual for phase contrast images. In the HDR color reconstruction (c) and in the black and white conversion (d), fine details within the cell and the nucleus appear with improved distinctness and resolution.

Figure 5 shows a detail from the epithelial cell of Figure 4 taken in (a) normal phase contrast, (b) reconstructed by Photomatix Pro, and (c) converted to black and white. At this higher magnification, the HDR reconstructions (b and c) provide better contrast, sharpness, and resolution; they show more details than are visible in normal phase contrast. According to our practical experience, all reconstructed images were either completely free of haloing and blooming, or the halo and blooming effects were significantly reduced. Thus, very-fine and low-contrast details remain visible in enhanced contrast and clarity.

Whereas conventional phase contrast is not very suitable for examination of stained or colored specimens, bright-field contrast amplification can be successfully used for a high-grade visualization of stained structures. When compared with normal bright-field mode, image reconstructions using the present method often showed more detail and structural nuances in such specimens, especially in regions with a high local density. As demonstrated in Figure 6, fine structures within the stained section of a pine leaf are just barely visible in normal bright-field (Figure 6a) because of the high optical density of this specimen. The HDR reconstruction (Figure 6b) shows more detail within the high-density regions, and the contrast is equalized over all parts of the specimen.

The major characteristics of conventional phase contrast and bright-field-based ultra-high contrast amplification are

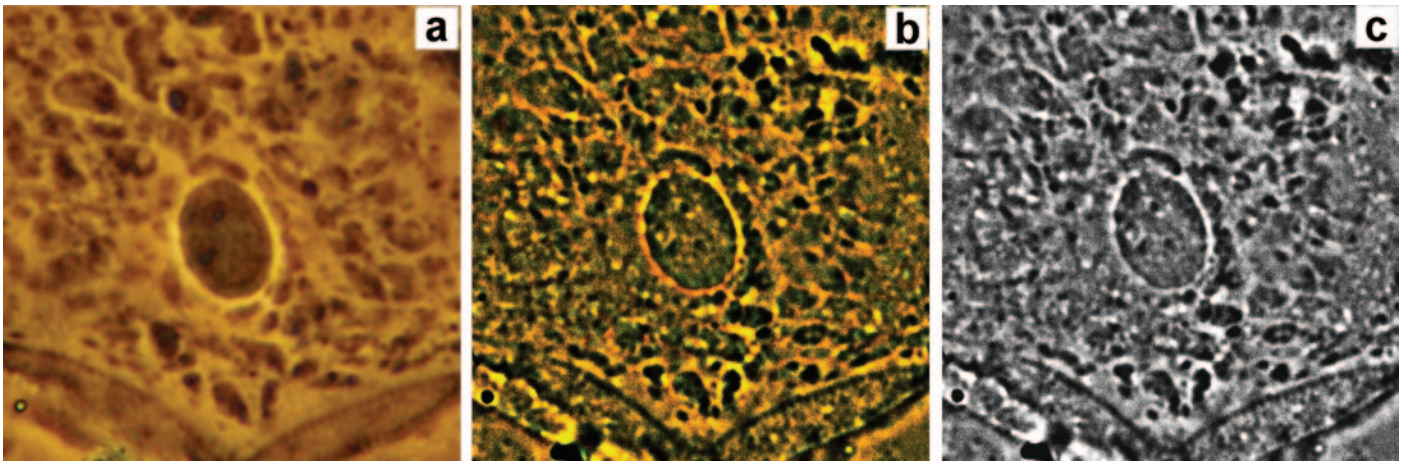

Figure 5: Detail from Figure 4 showing several subcellular structures in usual phase contrast (a), contrast amplified bright-field (b), and black and white conversion (c). Objective 40×, ocular 12.5×, image width = 30 µm.

Table 2: Characteristics of conventional phase contrast and ultra-high contrast amplified bright-field.

Key Features	Phase Contrast (Usual Technique)	Ultra-high Contrast Amplified Bright-field
Phase rings necessary	yes	no
Use objectives from all manufacturers	no (phase rings are specific for manufacturers)	yes (normal bright-field lenses can be used)
Condenser setting	phase contrast	bright-field
Condenser aperture diaphragm	fully open	smaller or closed (as usual in bright-field)
Contrast	good	higher
Contrast trimming	no	yes
Depth and planarity of field	narrow	higher
Resolving power	good	higher (when aperture diaphragm is wide open), sometimes lower (when aperture diaphragm is maximum closed)
Halo artifacts	yes	no (or less)
Brightness of the microscopic image	low	high

compiled in Table 2. When Photomatix Pro is used, the time needed for image rendering is dependent on the hardware resources and the image size. Table 3 and Figure 7 show time measurements for several image sizes based on an up-to-date computer (core quad processor 2833 MHz, 4 GB RAM, graphic card 512 MB, Windows XP).

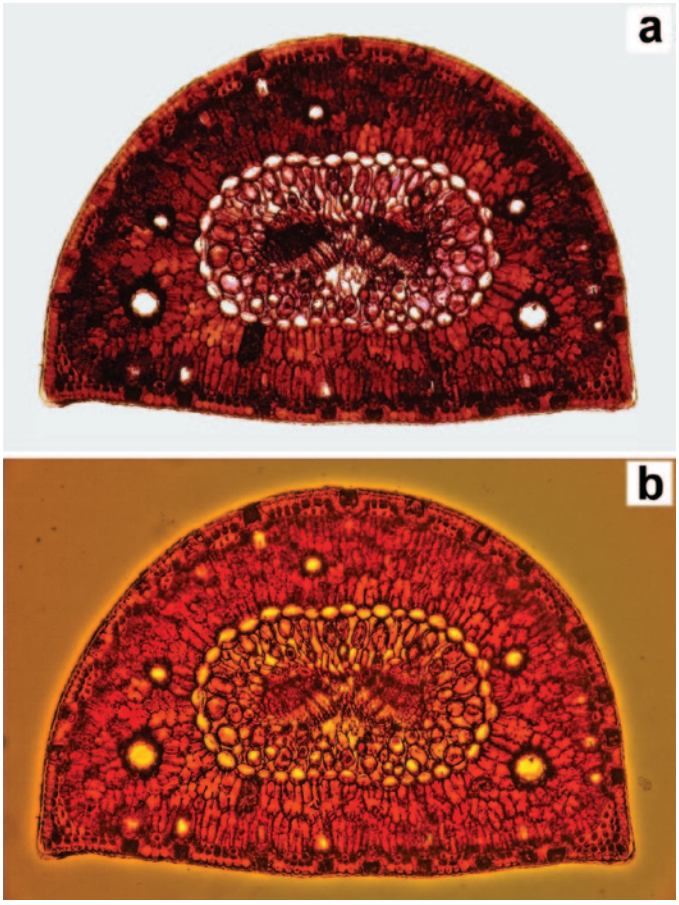

Figure 6: Leaf of a pine, stained section. Normal bright-field, filtered in ideal white (a) and contrast-amplified bright-field with equalization of brightness in specimen and background (b). Objective 4×, ocular 8×, image width = 1.5 mm.

Discussion

When HDR rendering is carried out, all details visible in concurrent prior techniques (bright-field and phase-contrast) are also visible in ultra-high contrast amplified bright-field imaging. However, these details are visible with higher quality. Well-known artifacts associated with phase contrast (blooming, haloing, loss of resolution, loss of deep and sometimes loss of planarity of field) are reduced or eliminated by the new technique. These artifact reductions and positive effects can be observed in the reconstructed images. When the new method is appropriately carried out, no new artifacts arise.

In all fields of imaging science, it is a serious legal issue to evaluate a new technique by comparing it by visual means with pre-existing methods. In general, this evaluation procedure takes place in three steps: First, the respective "object" or specimen is examined and documented by well-known and well-evaluated techniques that already exist (bright-field and phase contrast in the present article). Second, the same object or specimen is examined and documented by use of

Table 3: Time needed for HDR image processing for images of various sizes.

Image Size	Time for HDR Rendering	Time for Tone Mapping	Total Time
3072 × 2304 (7.1 MP) (20 MB TIF, 4 MB JPG)	5 sec.	15 sec.	20 sec.
2703 × 2027 (5.5 MP) (16 MB TIF, 3 MB JPG)	3 sec.	11 sec.	14 sec.
2181 × 1635 (3.5 MP) (10 MB TIF, 2 MB JPG)	2 sec.	7 sec.	9 sec.
1920 × 1080 (2.1 MP) (6 MB TIF, 1 MB JPG)	1 sec.	4 sec.	5 sec.
1440 × 1080 (1.5 MP) (4 MB TIF, 1 MB JPG)	1 sec.	3 sec.	4 sec.
1280 × 720 (0.9 MP) (3MB TIF, 0.5 MB JPG)	1 sec.	2 sec.	3 sec.

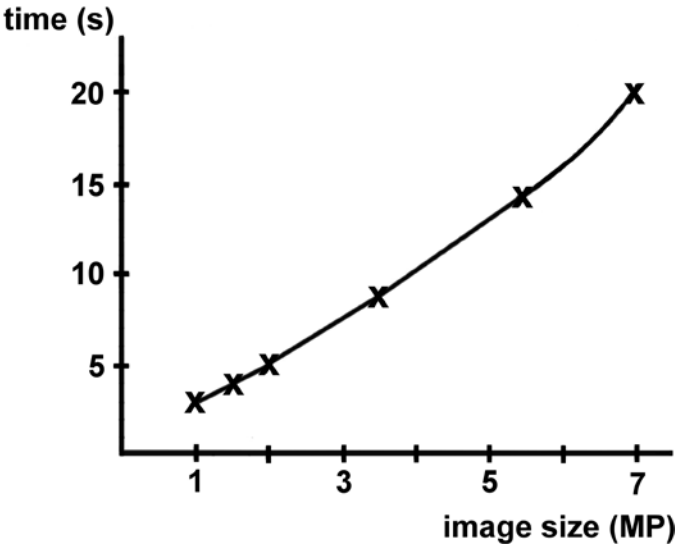

Figure 7: Image processing times (HDR rendering and tone mapping) for various image sizes (further explanations in the text).

the "new" technique (ultra-high amplified bright-field, based on HDR rendering). In a third step, both images—the prior and the new one—are compared with each other, so that the new method is evaluated in concurrence with well-evaluated prior techniques. When the visual information obtained by the new method seems to be equal or better than that of the prior techniques, the new method will be regarded as a positive further development, leading to better or superior results than achievable by the prior methods. In this manner, microscopists can learn how to interpret the results from new techniques in the context of established methods.

All in all, the described techniques in HDR imaging and additional multi-step post-processing can be regarded as attractive tools for visualization of unstained phase structures in transparent specimens of low optical density as well as in stained or colored specimens with high density. When compared with common phase contrast, the HDR bright-field images often show a higher grade of visual information. Halo artifacts are strongly reduced, and the condenser aperture diaphragm can be used for improvements of image quality in the same manner as in common bright-field microscopy (especially for enhancements of contrast, contour sharpness, vertical resolution, and planarity of field). Of course, in routine applications, unstained specimens should be examined and photographed in traditional phase contrast as in the past, and stained or colored specimens should be examined in ordinary bright-field as well.

The new method described here could lead to improved results in special purpose observations, for example, live-observations of cell cultures in small volumes. A new generation of improved digital live observation microscopes could be created if these techniques were integrated into a software-based workflow for image processing in real-time microscopy. Bright-field images could be directly detected by a suitable CCD camera equipped with a high-resolution sensor. When this camera generates 30 single frames per second, for instance, each pair of two consecutive images could be superimposed and processed to create an optimized HDR image within the refresh rate period. Thus, 15 HDR images could be created per second so that native living cells and other suitable specimens could be directly observed by this new technique. All technical and optical advantages presented above could be used for digital live microscopy of native motile specimens. Further technical developments could aim toward real-time trimming of several quality-determining parameters in such HDR-based image sequences whereas contrast enhancement and other parameters (for example, gradation and brightness) might be manually adjusted during live examination. In this respect, the new techniques described here should be of interest to manufacturers engaged in the development of new technical solutions for digital live microscopy.

Recipe for HDR Imaging in Three Steps with Photomatrix Pro

Step 1:
Two or more bright-field images are taken as usual, preferably at different exposures.
or
One single shot bright-field image is duplicated into two identical images.

Step 2:
Start Photomatix Pro
Menu "HDR" → "Generate"
Load different images ("Browse . . .")
Align source images
Take tone curve of color profile (recommended)
"OK" → HDR image is created
Menu "HDR" → "Tone Mapping"
Method: Details Enhancer
Adjustments (see preview image for visual control):
Strength: 100
Light smoothing: High
Color saturation: Low or zero (B&W)
Luminosity: Medium (in most cases)
"OK" → Menu "File" → "Save as . . ."

Step 3:
Conventional post-processing on demand
→ Optimization of gradation, histogram, contrast, brightness, color parameters, etc.

Conclusion

Visual information in bright-field images can be significantly enhanced by high-dynamic-range rendering (HDR rendering). Satisfying results from specimens of low optical density can be achieved when the original images are processed in a well-defined multi-step procedure: (1) an HDR-image is created, (2) a tone mapping procedure is carried out, and (3) the resulting reconstruction is further optimized with regard to its gradation, contrast, and brightness. Conversions to black and white are recommended, especially for images showing unstained colorless specimens.

These image reconstructions can be of higher quality than normal phase contrast images because: (a) The optical quality

of the objective lens is not degraded by a phase ring. (b) The aperture of the condenser is not reduced by an annular-shaped light mask. (c) The condenser aperture iris diaphragm can act in the same manner as in traditional bright-field imaging so that the depth and planarity of field, the contour sharpness, and the contrast in low-density specimens can be regulated in tiny steps. By digital image processing, in particular by tone mapping and final adjustment of gradation, image contrast can be optimally adjusted for a particular specimen.

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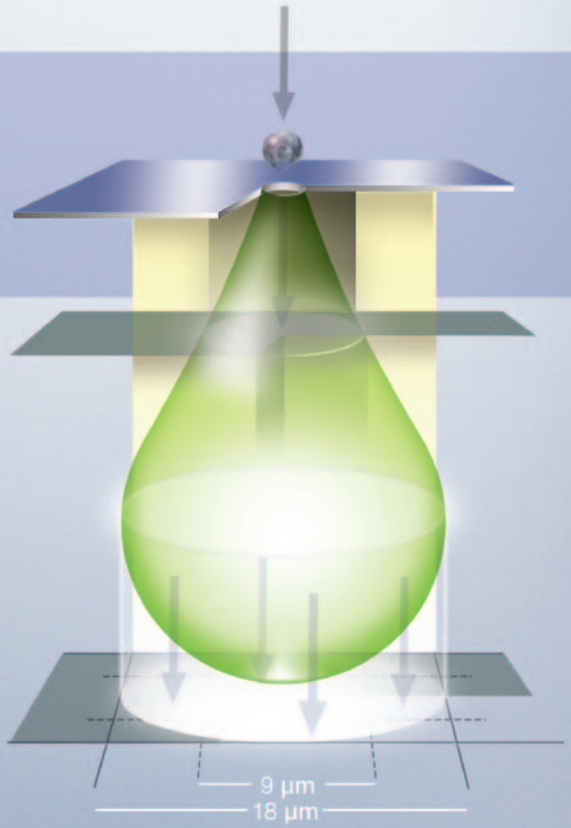

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