Software-based three dimensional reconstructions and enhancements of focal depth in microphotographic images

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Software-based, the focal depth in light microscopic photographic images can be enhanced fundamentally. Thus, three dimensional images are achievable, comparable with scanning electron microscopic results. Moreover several parameters which determine the quality of microscopic images can be improved by averaging. For these purposes, a vertical stack has to be taken as sequence or scan, consisting of several single images with different planes of focus. The single images are software-based superimposed; only regions, which are in focus, contribute to the resulting reconstructed image. Resulting images are without any unsharpness, with independence from the vertical dimension of the specimen or the magnification and focal depth of the objective.

Keywords three dimensional reconstruction; focal depth; sharpness; depth of field; stack; scan; averaging; post-processing; image; quality; improvement; photomicrography

1. Introdution

Microscopic images are characterised by a low focal depth. The higher the magnification of an objective, the smaller is the achievable depth of field. When high magnifying objectives are used, the focal depth the can be less than a wavelength of light. Table 1 presents the focal depth of microscopic objectives with regard to their magnification and numerical aperture [1]. When the magnification increases, the remaining focal depth decreases over proportionally.

Thus, it is often impossible to obtain sharp focus over the full depth of a specimen. The limitation of focal depth can be regarded as a general limitation of conventional photographic documentations in microscopy.

The three dimensional structure of a specimen can only be documented by conventional means, when a video clip is taken instead of a still image, while the stage is moved slowly. Alternatively, a still image can be taken with a lower magnifying objective, when the region of interest is cut out as a sectored image by post-processing.

Both methods have their specific limitations. Video techniques lead to low resolution images in comparison with photographic still images. Moreover, a video clip demonstrating three dimensional structures can be seen, but not be printed as one single image. Photographs taken with lower magnifying objectives are characterised by a lower resolving power in comparison with higher magnifying lenses.
Therefore, other methods are desirable to create high resolution microscopic images with maximized focal depth and optimized resolving power.
This aim can be achieved by various software solutions which are described and compared with each other below.

2. Materials and Methods

Several software solutions for image post-processing were tested based on digital images taken from various objects which were examined in different magnifications and illuminating modes by stereo and laboratory microscopes. Findings of other authors were additionally taken into account.

On detail, the following software was considered: Combine Z 5 [2], Helicon Focus [3], AutoMontage [4], Registax [5], Astrostack [6].

Combine Z 5, Helicon Focus and AutoMontage have been developed as software solutions for three dimensional reconstructions of high resolution still images. Registax and Astrostack were generated for astrophotographic purposes to achieve improved still images from video clips; they are usually regarded as tools for reduction of noise and other artefacts.

To obtain a digital enhancement of depth of field, the stage of the microscope has to be moved up or down in tiny steps, so that the plane of focus passes across the specimen. By doing this, a vertical stack or sequence of images can be achieved, each with a very shallow focal depth, which is dependent from the magnification of the objective. Thus, only a small part of the respective image is in focus. The higher the magnification and the lower the focal depth, the higher is the required number of single images to achieve a complete vertical scan of the specimen without zones of unsharpness.

The sequences of single images have to be loaded as image files by the respective reconstructing software. Afterwards, all single images are software-based modified in this order: colour saturation, white balance and brightness are re-adjusted, artefacts are reduced, all images are properly aligned, so that their congruence will be maximized. Finally, the software combines the in-focus parts of each of the planes in the stack into a resulting completely in-focus image. Three dimensional reconstructed images can be saved as separate image files and processed by other conventional software, if necessary.

The software solutions, tested out, were evaluated comparatively using sequences of high resolution still images taken by digital cameras (Canon Powershot A 95, Olympus Camedia C 7070). Video clips were not tested, because high resolution still images will lead to higher resulting qualities.

3. Results

The software solutions, mentioned above, lead to different qualities of three dimensional reconstructions, because they work with different algorithms. The basic results of comparative tests and the main features of the respective software are presented in the following context:

Combine Z 5

This freeware leads to excellent results in photomicrography based on its presets. When the single images of the respective stack are loaded, the image processing can be started by several macros:

„Do Stack“ is the standard macro for most purposes, leading to very good results in most cases.

„Do Average and Filter“ is an alternative macro which produces reconstructions with enlarged contrast and sharpness. This macro can be advantageous in some situations.

„Stack Only“ creates superpositions of single images without further corrections or alignments. This macro can be used for the processing of images which are properly pre-aligned when existing high local differences in brightness and contrast are to be averaged.

When the resolution of the single images is 3 megapixels or less, the frames of the single images and the resulting reconstructed image are shown in full screen mode, while the software works.

When the resolution is higher than 3 megapixels, the progress of the image processing is just presented in text mode. The resulting reconstructed image has to be saved as a separate image file and can just be evaluated afterwards by usual viewer software.

Two macros can be used for data saving, „Save Frame/Picture As“ or „Save Rectangle As“. Both macros lead to nearly identical results when the original image is saved in full size. Occasionally, the focal plane of focus differs marginally, when an image is simultaneously saved as frame/picture and rectangle. In this case, the final sharpness of the resulting image can be additionally enhanced when both reconstructed images (frame and rectangle) are stacked again in a second step (double stacking).

In some cases, Combine Z 5 does not work successfully when a specimen is illuminated by monochromatic light.

Helicon Focus

This software can be evaluated as shareware; all features can be unlimitedly used over a period of 30 days. The price for license is about $ 120 (basic version) or $ 250 (advanced version).

When the existing presets are used, this software leads to results which are completely comparable with those of the macro „Do Stack“, implemented in the software Combine Z 5.

The single images and the resulting reconstructions are not presented in a full screen mode, but in a much lower sized window. On the other hand, the progress of image processing and the resulting reconstructed images are always presented in live view mode, also in situations, when the resolution of the single images is higher than 3 megapixels; tests were carried out successfully up to 8 megapixels. Monochromatic images can be averaged with high accuracy, too.

AutoMontage

This software was not tested out by the author because of its high price (about $ 1000). According to otherwise findings, AutoMontage can be regarded as an excellent tool for professional three dimensional image processing, characterised by a lot of features [1]. On the other hand, the quality of achievable three dimensional reconstructions seems to be comparable with Combine Z 5 [1].

Astro software

Registax is able to superimpose still images up to a resolution of about 2 megapixels. The software does not run when the resolution of image files is higher. Moreover, the correctness of alignment is worse, when microscopic images are processed. Astrostack has been evaluated by another author [1]. According to published results, the quality of three dimensional microphotographic reconstructions is lower, too, when this software is compared with Combine Z 5 and AutoMontage. It might be taken into account, as a potential reason for this that this software has been developed for astrophotographic video processing.

Quality of three dimensional reconstructions

According to own experiences, excellent three dimensional reconstructions are achievable when Combine Z 5 or Helicon Focus is used. In all situations (stereo microscopy, conventional light microscopy) the respective singe images are aligned and averaged very exactly. The high quality of reconstructions is constant over the full range of microscopic magnification. The number of single images, necessary for good results, ranges from circa 4 to about 20 or 30, dependent on the three dimensionality of the object and the magnification or focal depth of the objective.

When a number of single images are superimposed, a 3 megapixel resolution already leads to very good results, comparable with at least 6 megapixel still images.

The sizes of reconstructed images are higher than those of a corresponding single image, because reconstructed images have more visual information; their size can be about two or four fold higher, determined by the number of single images and the structure and details of the specimen.

The figures demonstrate the high quality of reconstructed microscopic images, processed with the help of Combine Z 5 (fig. 1-3) and Helicon focus (fig. 4-5). Each figure shows arrangements of images, consisting of a representative conventional single image and corresponding three dimensional reconstructions.

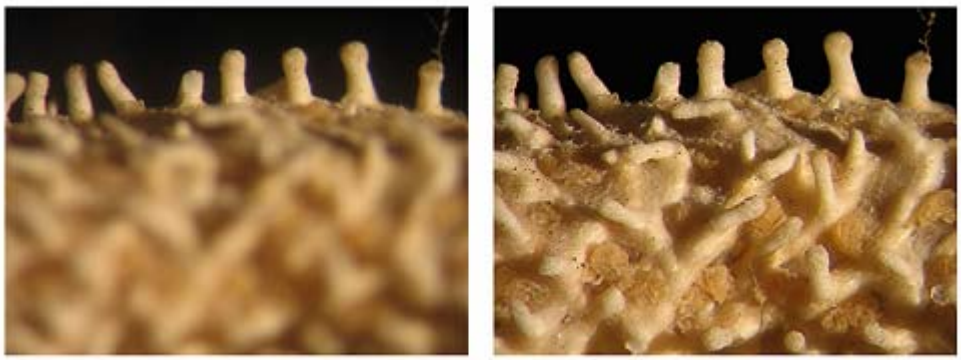

Fig. 1: Asteroid-arm, stereo-microscope, epi-illumination, objective 2x, ocular 10x, horizontal field width (HFW): 4 mm. Superposition of 8 single images, double stacking (software: Combine Z 5).

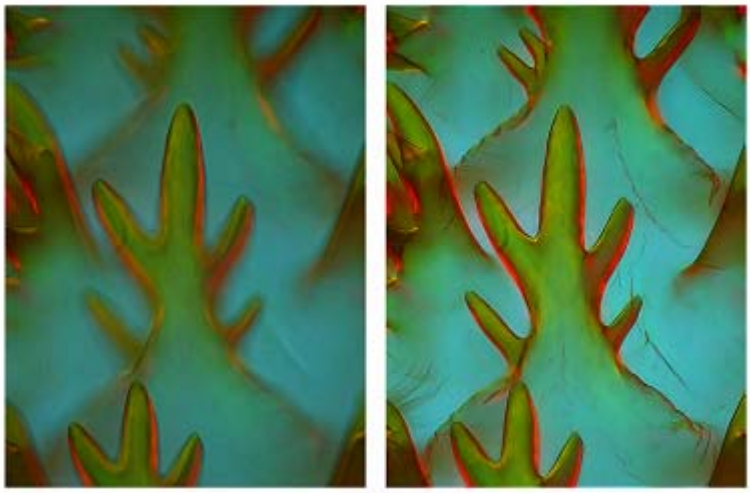

Fig. 2: Stomach of a cricket, detail, polarization, additional lambda compensator, objective 40x, ocular 8x. Horizontal field width (HFW): 0,15 mm, vertical field width (VFW): 0,2 mm. Superposition of 14 single images (software: Combine Z 5).

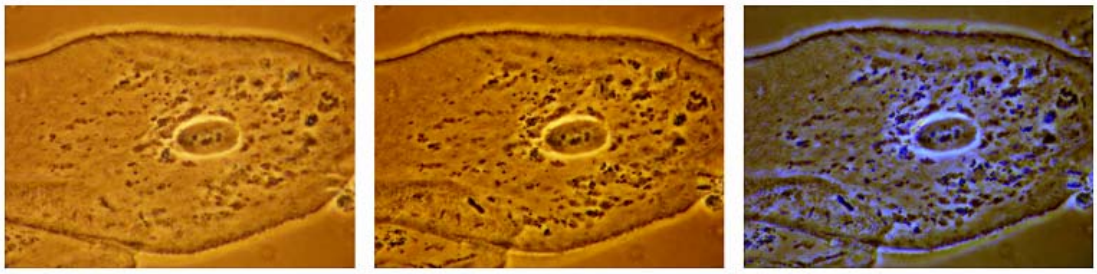

Fig. 3: Human epithelial cell from the oral mucosa, live-preparation, phase contrast, objective Oil 100x, ocular 12,5 x. Horizontal field width (HFW): 0,05 mm. Superposition of 11 single images and colour sandwich technique (software: Combine Z 5).

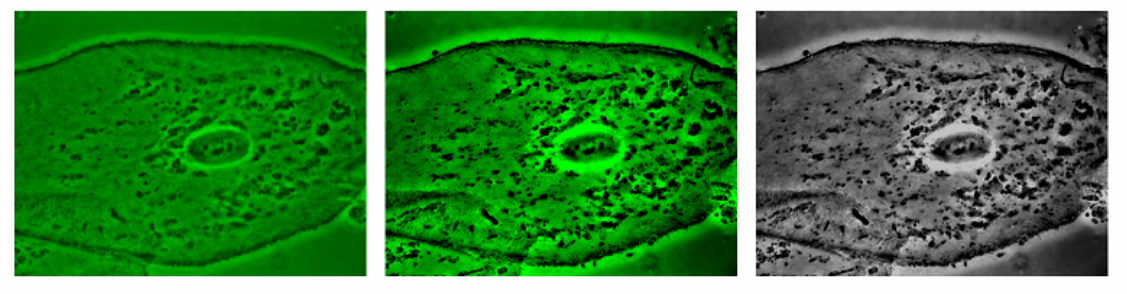

Fig. 4: Human epithelial cell from fig. 7, phase contrast, monochromatic green light, objective Oil 100x, ocular 12,5 x. Horizontal field width (HFW): 0,05 mm. Superposition of 11 single images (software: Helicon Focus).

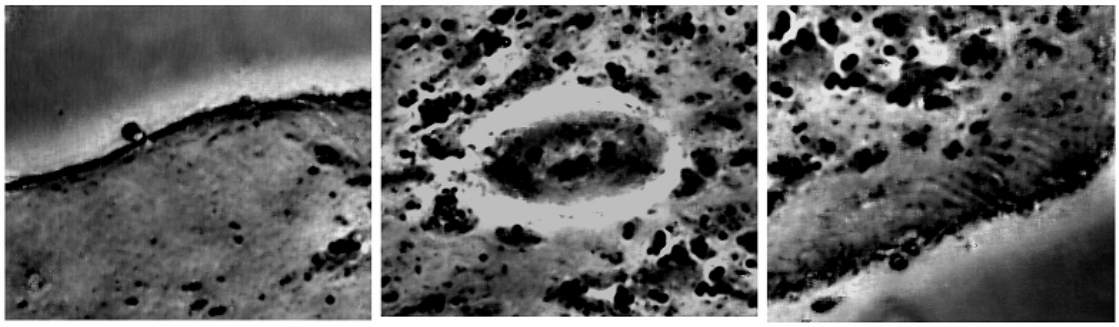

Fig. 5: Details from fig. 8, sub cellular structures (textures of cell membrane and nucleus). Horizontal field width (HFW): ca. 0,01 mm.

Software-based reconstructions of three dimensional objects create extraordinary improvements of focal depth, sharpness and visual information; potential artefacts and local imbalances of brightness and contrast can be reduced.

Special techniques for reconstruction
In some situations, special methods can be taken into account for further improvements of the resulting three-dimensional reconstructions.

Double stacking:
In special cases, the resulting quality of reconstructions can be additionally improved by „double stacking“, as mentioned above. For this purpose, a specimen has to be stacked twice; the reconstructed images from both stacks have to be stacked again (fig. 1).

Colour sandwiches:
Double stacking can also be used for colour sandwich techniques, when both sequences are taken in different illuminating light. Fig. 3 shows an example of a colour sandwich in phase contrast. One sequence was taken in electric bulb light, a second identical sequence in flash light. As the colour temperatures are different, a new character of colour contrast occurs so that fine structures, especially at the margin of the cell membrane, are accentuated. The achievable effects are similar to solarization, caused by the coincidence of red-yellow bulb light and blue flash light.

Monochromatic light
Monochromatic green light (wave length: 540 nm) can lead to optimized sharpness, because potential chromatic aberrations are completely eliminated. Moreover, potential interpolation artefacts are

minimized in digital images, when green light is used. Therefore, monochromatic green light can lead to optimal results, when images are to be created in black and white (fig. 4 and 5).

Processing times for three dimensional reconstructions
Usually, three dimensional reconstructions are created within a few minutes after starting the respective software. When the size of single images is doubled, the processing time is at least doubled, too, in some cases nearly tripled. The processing time increases in proportion to the number of single images (frames).

Discussion

Low focal depth can be regarded as a fundamental limitation of each conventional photographic documentation of microscopic images. Especially objects with a great vertical dimension can often not been photographed in a satisfying manner.

Software-based techniques can lead to excellent improvements in three dimensional photomicrography. In principle, the depth of field can be maximized infinitely, when the scanning mode is carried out adequately.

Within the range of light microscopic magnification, the resulting images can be compared with those taken by scanning electron microscopes. It could be taken into account, as an advantage of light microscopic reconstructions that each specimen can also be photographed in its original colour instead of a monochrome documentation.

Moreover, potential unsharpness caused by differences in the accommodation of the human eye can be deleted, also in flat objects with a low vertical depth, when a stack of single images is superimposed, consisting of several images with low differences in their focalization.

As the processing time is just a few minutes and as the suitable software solutions work very sufficient, these techniques can be easily integrated in each photographic microscopic workflow.

References

[1] Hart, J.: 3D Microscopy and high-magnification macro-photography: Digital reconstruction and depth-of-field expansion. University of Colorado.
http://www.crystalcanyons.net/pages/TechNotes/3DMicroMacro.shtm, 2003
[2] Hedley, A.: CombineZ5. My software to combine pictures to increase depth of focus.
http://www.hadleyweb.pwp.blueyonder.co.uk/index.htm, 2006
[3] Danylo: Helicon Focus. http://www.heliconfocus.com/pages/index.php?focus_overview, 2006
[4] Synoptics Group, Syncroscopy: Auto-Montage. Unique software producing infinite depth in focus with additional 3D modelling and measurement features. http://www.syncroscopy.com/syncroscopy/products.asp, 2006
[5] Berrevoets, C.: RegiStax. Free image processing software. http://registax.astronomy.net/, 2006
[6] InnoStack: Astrostack. www.astrostack.com, 2006