Improvement of plant cell microscope images by use of ‘Depth of Field’ - extending software

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Abstract

Transmitted light and fluorescence microscopy often suffers from the limited Depth of Field (DOF) of microscope objectives. For this reason, plant cells can in most cases not completely be visualized in a single image at suitable magnification. In case of fluorescence signals, this problem can to some extent be circumvented by the use of confocal laser scanning microscopy (CLSM) which is able to remove disturbing light that is out of focus, thus leading to a clear image within the focal plane. Several of such confocal images can then be combined to provide a virtual image pretending a greater DOF. However, this trick is not possible for transmitted bright field microscopy, not even if CLSM operating in the imaging mode is applied. Instead, stacking software packages, which are based on algorithms capable of recognizing those parts of an image that are in focus, might provide an alternative approach to overcome this problem. Here we show that such software packages can be used to assemble mathematically the focal information of a z-stack of arbitrary microscope pictures into a virtual single image. This virtual image shows an enhanced DOF irrespective of whether the original data are epifluorescence or transmitted light microscopy images of plant cells and if they were obtained by conventional microscopy or CLSM.

This method thus provides an affordable alternative to confocal image analysis and a meaningful tool in plant cell imaging. Furthermore, this method is even suitable to visualize organelle movement within a single image.

Introduction

Transmitted light and fluorescence microscopy are important tools in plant cell biology that gained further importance by the introduction of fluorescent proteins (reviewed in Mathur 2007). One major drawback of conventional light and fluorescence microscopy is the limitation in Depth of Field (DOF), which is generally defined as the area behind and in front of the focal plain in which structures appear sharp. Areas out of the DOF are blurred, show low contrast and few details. The limitation of the DOF depends on the magnification of the objective used and becomes more limited with increasing magnifying power of the objective. Conventional microscope dry objectives exhibit a DOF ranging from less than one to only a few micrometers (Piper 2008).

Plant cells represent a particular challenge in microscopy, because the organelle-containing cytosol is displaced towards the periphery of the cell by the huge central vacuole. In consequence, it is essentially impossible to have all structures of a plant cell in focus at the same time. Focusing through the specimen overcomes this problem and thus gives an impression of the entire cell. However, documentation of this process is difficult, since series of printed images of different focal plains are often difficult to interpret.

Several possibilities are currently used to extend the DOF. They are all based on the collection of image series along the Z-axis (Z-stacks) representing a number of focal plains throughout the specimen. One approach is to overlay all pictures of a Z-stack using graphic
tools. The regions in each image that are out of focus are afterwards manually erased resulting in an image representing the focal information of the Z-stack. This method was successfully used, for example, to visualize GFP-tagged plastids in etiolated hypocotyl cells of *Nicotiana tabacum* (KWOK et al. 2003). However, due to differences in background fluorescence intensities within the Z-stack, such images sometimes appear mosaic-like. Furthermore, the application of this procedure to a more complex specimen (e.g. with higher numbers of organelles) would significantly increase the expenditure of time for the manual processing. One alternative is, therefore, confocal laser scanning microscopy (CLSM), which acquires fluorescence images containing signals exclusively from the focal plain. Confocal images taken by CLSM offer the possibility to obtain extended DOF images by a maximum projection based on the fluorescence Z-stack, but it has to be mentioned that the confocal microscopes required are expensive and thus not always available. Furthermore, the exclusion of blurred light by CLSM is only possible for fluorescent signals. Transmitted light images of CLSM (e.g. differential interference contrast = DIC) suffer from the same limitations concerning DOF as those obtained by conventional microscopy. Therefore, alternative techniques to obtain high quality images with extended DOF also from conventional epifluorescence and transmitted bright field data are desirable. The development of algorithms capable of recognizing those parts of an image, which are in focus, enabled the development of so-called "stacking" software packages. These programs are able to assemble the focal image parts of a Z-stack taken by conventional optics into an extended Depth of Field image (MENESES et al. 2008). In the last years, such stacking software packages became more and more important in photography, also for scientific purposes (e.g. in arthropology, mineralogy, paleontology and material science). At present, several packages are available (e.g. as modules of image acquiring software or stand alone solutions), which include even some software packages that are free of charge (summarized in PIPER 2008). We have exemplarily tested the suitability of the stacking program CombineZ5 (HADLEY 2006) to microscopic Z-stacks of plant cells (lower epidermis of *Nicotiana benthamiana* and upper epidermis of *Arabidopsis thaliana*) that were acquired by epifluorescence and DIC microscopy using both conventional and confocal laser-scanning microscopy.

**Methods**

**Fusion proteins, plant material and transformation**

For visualizing plastid morphology, *Nicotiana benthamiana* was stably transformed (HORSCH et al. 1985) with the FNR-EGFP construct (MARQUES et al. 2003; MARQUES et al. 2004). The respective coding sequence was inserted into the binary T-DNA vector pGreenII 0129 (HELLENS et al. 2000) downstream of the CaMV 35S promotor. For labeling Golgi vesicles with cyan fluorescent protein (CFP), *A. thaliana* was transformed by biolistic transformation (RUBERTI et al. 2006) with the G-cK construct (NELSON et al. 2007).

Both *N. benthamiana* and *A. thaliana* plants were grown under short day conditions. For microscopic observation, discs of 5 mm diameter were punched out of fully expanded leaves. In order to reduce thermal stress during microscopic observation, leaf discs were vacuum infiltrated and mounted in artificial pond water (YAMADA et al. 2003).

**Confocal laser scanning microscopy (CLSM)**

CLSM images were taken with a LSM 510 microscope (Carl Zeiss, Jena, Germany). CFP was exited by the 458 nm laser line of the Argon laser, while the DIC images were recorded using the 633 nm HeNe laser.
**Epifluorescence and DIC microscopy**

All DIC and epifluorescence images were taken with a non-motorized Axioplan 2 research microscope (Carl Zeiss, Jena, Germany) equipped with an Axiocam HRc CCD-Camera (Carl Zeiss, Jena, Germany) controlled by the manufacturer's acquisition software AxioVision. EGFP-fluorescence was visualized using the F36-525 filter set (AHF Analysentechnik AG, Tübingen, Germany).

**Image processing and stacking**

CombineZ, a free software package developed by Alan Hadley (HADLEY 2006), was used for processing Z-stacks. Three different program versions are available: CombineZ5, CombineZM and CombineZP. We decided to use CombineZ5, which will probably remain unchanged, in contrast to CombineZP, being the latest release, which is currently still further developed. In order to keep the processing as simple as possible, images presented in this paper are results of the preset "do stack macro" (keeping up the default settings). For further information about processing details, the reader is referred to the help function of CombineZ. The processing procedure performed by CombineZ, which leads to extended DOF images, is addressed here as "stacking".

For image acquisition and adjustments, the microscope and camera software provided by the manufacturer and Adobe Photoshop (San-Jose, CA USA) were used.

**Results and Discussion**

**General procedure**

In order to generate images with extended DOF, it is essential as the first step to acquire image series (Z-stacks) of the entire sample in which each structure of interest is at least once in focus. This is best achieved by stepwise focusing through the sample and taking images at each step. To realize reproducible stepping, a software-controlled motorized stage or piezo-stepper is ideal. However, manual focusing by an experienced microscopist is generally sufficient as well. Actually, all examples shown here were achieved by manual stepping, except for the CLSM images. In order to accelerate the manual stepping process, the time series function of the acquiring software was used.

The resulting Z-stack was subsequently converted into single images of standard file format, which can be imported by the stacking program. For the actual stacking procedure, different algorithms were applied by the stacking software to decide which image slice in the imported Z-stack is at a given position most likely in focus (MENESES et al. 2008). Afterwards, the chosen pixels are assembled into a single image, which represents a view with extended DOF. The resulting image can be exported and used for presentation purposes. The quality of the resulting image can further be improved by adjustment and correction of the image stack prior and after the stacking procedure (PIPER 2008). Such adjustments have generally to be evaluated for each specimen. However, the CombineZ5 package used in this study provides a set of default settings as "preset macro", which covers several suitable preprocessing (e.g. balance color and brightness) and postprocessing procedures (e.g. removing color steps). All images presented here were obtained with these default settings.

**DIC**

As first example to demonstrate stacking of DIC and epifluorescence images, the lower epidermis of transgenic *N. benthamiana* leaves expressing FNR/EGFP (MARQUES et al. 2003; MARQUES et al. 2004) is shown. Since almost the complete lamina is nerved by vascular strands of different size, especially the lower epidermis is often not plain and can thus not be visualized in a single image. Using DIC settings, a Z-stack throughout the epidermal layer was recorded and finally stacked. In contrast to the single images of the Z-stack, which
show only a small area in focus (Figures 1 A, B, C), the resulting stacked image represents the full information of the whole Z-stack in a single image (Figure 1 D). The lateral cell walls of each cell, which mark the cell boundaries in the observed area, are precisely displayed. In addition, almost all plastids and nuclei of the epidermis cells are clearly visible.

**Epifluorescence**

The same leaf section was analogously analyzed with regard to EGFP fluorescence (Figure 2). The bright, spot-like fluorescence signals in the single Z-stack images (Figures 2 A, B, C) correspond to the EGFP containing plastids of the lower epidermis. Images in which the focal plain crosses the spongy pa-

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**Figure 1:** DIC images of lower epidermis cells of transgenic *N. benthamiana* expressing FNR/EGFP; Examples of (A) upper (1th), (B) middle (7th) and (C) lower (12th) images of the Z-stack. (D) Stacked image representing the focal information of all images of the Z-stack. Note that cell walls, plastids (p) and nuclei (n) of all observed cells are visualized simultaneously in focus. (scale bar 50 µm)

**Figure 2:** EGFP epifluorescence images of lower epidermis cells of transgenic *N. benthamiana* expressing FNR/EGFP; Examples of (A) upper (1th), (B) middle (10th) and (C) lower (20th) images of the Z-stack. The leaf sector shown is identical to that analyzed by differential interference contrast microscopy as shown in Figure 1. Note that in the stacked image (D), all epidermal plastids (p) are visible and show a clear outline. (scale bar 50 µm)
renchyma (e.g. Figures 2 B, C) show dimmer but larger signals representing chloroplasts. Plastids from above and below the focal plain cause blurred and round signals (Figure 2 C) resulting in a smooth background fluorescence (Figure 2 A). Due to internal light reflections, the cell borders are highlighted in some areas. Like in the DIC images, only few details (organelles) are visible in the single images. In contrast, in the stacked image (Figure 2 D) all epidermal plastids are presented as spot-like fluorescence signals with a clear outline. The fuzzy chloroplasts of the spongy parenchyma are displayed as well but they do not interfere with the different signals of the epidermal plastids. The stacked image is further characterized by reduced background fluorescence that is caused by plastids that are out of focus (compare Figures 2 C, D). This is not only the result of the stacking algorithm but might also be due to the pre- and postprocessing procedures included in the "do stack" macro (see above).

Confocal laser scanning microscopy (CLSM)

CLSM is able to prevent any disturbance by fluorescence light that is out of focus. As a consequence, extended DOF can easily be achieved from fluorescence Z-stacks by maximum projection, which is included as a feature in CLSM user software. However, the corresponding transmitted bright field images suffer in the same way from out of focus structures as images from conventional microscopy do. In order to obtain extended DOF images for a meaningful overlay with fluorescence projections, DIC CLSM Z-stacks were processed

Figure 3: CLSM images of a transiently transformed A. thaliana leaf epidermal cell expressing CFP targeted to Golgi vesicles (NELSON et al. 2007); Examples of (A) upper (1st), (B) middle (5th) and (C) lower (9th) images of the Z-stack. In contrast to the single images, the stacked image (D) displays almost the complete outline of the transformed cell and shows details and an overall sharp appearance. In contrast, the maximum projection (E) generally applied to CLSM fluorescence images does not provide that many details. In the overlay of the stacked image with the CFP fluorescence maximum projection (F) it is visible that the labeled Golgi vesicles are found exclusively in one cell, while neighboring cells lack any fluorescence signals demonstrating that they were not transformed. Black dots at the cell surface correspond to residual gold particles from the biolistic transformation process. (scale bar 10 µm)
as described above for images achieved with a conventional light microscope. In the example shown here, CLSM images of a transiently transformed *A. thaliana* epidermal leaf cell expressing a CFP fusion protein accumulating in Golgi vesicles were used (Figure 3). Due to the limited DOF, neither of the bright field images in the Z-stack visualizes the complete cell outline preventing a meaningful overlay of these images with the fluorescence projection. However, after stacking the cell outline is nicely visible (Figure 3 D).

**Image processing artifacts**

In spite of the general suitability of the stacking procedure, it should be noted that it can lead in some instances also to artifacts which can cause misinterpretations. For example, since most plant cells are microscopically "transparent" structures, organelles at the top and the bottom of a cell which are located at the same x/y-position but at different z-positions might eventually be put in focus within a single Z-stack. In this instance, details of both organelles might artificially be combined in a single structure in the subsequent stacking process.

The resulting image would then be confusing and misleading. One example is shown in Figure 4 B in which chloroplasts from the spongy parenchyma are combined with the cell wall of the covering epidermal cell. One way to overcome such problems is to manually blur those regions in the single images which shall not appear in the stacked image so that these structures will be of lower priority for the software during the stacking process. In the example shown in Figure 4 B', the chloroplasts of the parenchyma cells were manually blurred by this method.

![Figure 4: Examples of apparent processing artifacts; (A) Part of the stacked DIC image (Figure 1) showing examples of apparent processing artifacts. In (B), the section of the stacked image, which was chosen for manual image improvement, is shown. In (B'), the artificially fused chloroplasts and cell wall were cured by manual blurring of a subset of the images. Section (C) shows the apparent double cell wall generated by the stacking process. In (C') and (C''), the corresponding single images are shown. For further details see text. (black scale bar 50 µm; white scale bars 12.5 µm)](image_url)
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The resulting stacked image therefore lacks any confusing chloroplast details, whereas the cell wall, which was partially suppressed in the previous stacking (Figure 4 B), is now completely visible. A second typical stacking artifact is caused by the often irregular curvature of the lateral cell contact surfaces. As a consequence, lateral cell walls sometimes appear at different positions in the various images of the Z-stack (Figures 4 C’, C’’), which leads to a stacked image showing an apparent "double" cell wall (Figure 4 C). However, since this apparent duplication of the cell wall is a consequence of the extended Depth of Field, it is actually not a processing artifact. This demonstrates that for the final interpretation of such stacked images the original Z-stack has always to be consulted.

In general the topics discussed are also relevant to epifluorescence images, but in the examples presented here neither "interfering sharpness" nor "double cell wall" phenomena have been observed. Nevertheless, it might play an important role in other instances. Therefore, the suitability of image stacking has to be critically proven for each given Z-stack and the stacked images have to be carefully screened for possible stacking artifacts.

Organelles in motion

Plant cell organelles exhibit remarkable mobility (WADA et al. 2004), e.g. movement along the cytoskeleton. Such movement fulfills a variety of cellular functions including distribution of organelles, stress adaptation (WADA et al. 2004), and driving the hydrodynamic flow.

Figure 5: DIC images of vesicle-like moving organelles of an A. thaliana leaf epidermal cell; Sample images of two time series are shown. The arrowheads mark the positions of an organelle at different time points (Red = 1st, black = 10th, blue = 20th image). A’ - A’’’ are sample images of a time series with 0.18 s step size (A’ = 0 s; A’’ = 1.65 s; A’’’ = 3.48 s). In the corresponding stacked image (A-1), the organelle track is visible as a row of tightly dotted spots. Deleting every second image extends the step size to 0.36 s, which results in a better resolution (A-2). B’ - B’’’ are sample images of a time series with 0.39 s step size (B’ = 0 s, B’’ = 3.64 s, B’’’ = 7.70 s). In the stacked image (B-1) variable distances between the single organelle dots indicate altered velocity in the motional process. Also in this case, the organelle movement is better resolved by deleting every second image (increasing step size to 0.78 s) (B-2). (scale bar 5 µm)

Visualizing organelle movement is generally based on time-lapse movies. For printed presentation, image series are commonly used. Alternatively processing by special organelle tracking and velocity software is nec-
necessary (AVISAR et al. 2008; SPARKES et al. 2008). In order to demonstrate how organelle motion can be visualized in a convenient way by using stacking software, two time series of the upper epidermis of A. thaliana were recorded and subsequently processed by the stacking macro (Figure 5). During the stacking process, the moving organelle is addressed as a group of independent structures by the stacking software, and therefore placed separately into different positions within the stacked image. The resulting image thus shows the mobility of the organelle as a "dotted organelle track" (Figure 5). In addition, information about the relative velocity of the organelle movement can be deduced from the image, because larger distances correspond to faster movement.

One prerequisite for the successful stacking in this case is a blurred background to avoid interfering structures of background and organelle. Furthermore, the optimal step size has to be evaluated in each case. If time steps are to short the stacked image will not properly resolve the organelle track (Figure 5, A-1, B-1). Still, we recommend short time steps for recording, because stacking might be done also with a selection of images only (e.g. every other image only) (Figure 5, A-2, B-2). Thus, stacking of a suitable time series of images showing a moving organelle can demonstrate both its track and its dynamics.

Conclusions
DIC and epifluorescence microscopy cover a substantial part of contemporary cell biology methods. However, already since the development of the first microscopes, microscopists have struggled with the limited DOF provided by the objectives used. In addition to the possibility to extend the DOF by fluorescence maximum projections using modern CLSM or other technical devices like optical grids, stacking software seems to be a promising tool to improve images even of conventional transmitted light and epifluorescence microscopes. Using sample Z-stacks and software packages similar to CombineZ5, which was exemplarily analyzed here, it is easily possible to obtain extended DOF images, which are useful substitutes for the commonly used image series. Because the Z-stacks can be acquired by conventional DIC and epifluorescence microscopes without the need of special equipment, the use of stacking software is a suitable alternative if CLSM is not available.

Stacking programs like those applied here are not restricted to extending the DOF of microscopic images. The flexibility of stacking software is demonstrated by their suitability to visualize also organelle motility and dynamics in single images, which offers an alternative to commercially available organelle tracking and velocity software packages. However, it is important to keep in mind that stacked images are virtual images and only the result of algorithms, which do not consider the biological background of the processed images. Therefore, depending on the specimen used, images might result, which do not represent true information but are only artifacts of the stacking process. This applies particularly to microscopically transparent objects like plant cells in which different structures can be seen at the same x/y-position within a Z-stack which causes interference during the stacking process. Therefore, interpretation of stacked images requires great care and should always refer to the original dataset.

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