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Advanced Imaging Techniques: Combining Focus Stacking and Image Stitching in Biological Photomicrography

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Abstract
The usual way of getting pictures in bioimaging involves a variety of optical tools. However, this method is limited by a shallow depth of field and a small view of field, which makes the shapes of plants and the tiny structures of insects unclear. Conventional methods are unable to show accurately the three-dimensional specimens, leading to poor image quality and wrongly judging the depth. The aim of this work is to combine focus stacking and image stitching methods to make large mosaics and high-resolution composites having micro details of morphological and anatomical features for useful for biological studies. For this high-definition images were acquired using a Nikon D5600 DSLR camera fitted with Laowa 2.5–5X lenses and Plan microscope objectives. Helicon Focus and Zerene Stacker were used for focal stacking, and Adobe Photoshop was used to combine the exposed layers put together in panoramic mosaics. In order to create large, high-resolution composites, post-processing techniques were used to aligned overlapping photos and distortions were eliminated. By employing a combiner, were produced extensive, high-resolution images characterized by enhanced depth of field and improved light propagation, thereby facilitating precise mapping of cellular layers and surface elements over expansive areas. The detailed and complexity of panoramic mosaics are better than those of single-shot micrography, and the short coming traditional photomicrography are reduced. By combining focal stacking and stitching techniques, it is possible to reliably image large 3D specimens, which will help in studying microorganisms, plants and animals very highest accuracy for quality researches.

Keywords: Focus stacking, Image stitching, Photomicrography

INTRODUCTION

High-resolution photomicrography is an important tool for scientists studying plants and insects because it lets them see how cells are organized [1]. Visual data from high-resolution imaging is important for scientific studies because it lets researchers make consistent data for taxonomy, morphological and anatomical studies [2]. The progress of biological research depends on scientists being able to see the detail of plant and animal tissue [3]. Biological specimens are a great challenge for traditional microscopy. Images made with traditional methods have a shallow depth of field and viewing restrictions few parts of

the specimen go out of focus and parts don't fit together [4, 5]. These limitations create analysis barriers, especially in intense research studies, as critical aspects can lie beyond the camera viewing areas [6, 7].
Transformative imaging methods like focus stacking and image stitching now solve all the difficulties in microscope imaging. Active researchers put together several frames from different focal planes to make a single structural image with a clear depth of field that shows the three-dimensional object clearly [8]. This method proves useful for scientists studying botany, zoology and microbiology, as it produces distinct images of layered materials [9, 10]. Researchers use the resolution-friendly panorama creation technique during image stitching processes for working with overlapping images [11, 12]. By getting rid of both spatial image limitations and depth constraints, these combined methods make it possible to analyse complex specimens in a planned way [13].
The study helps combining focus stacking technology with image stitching tools making it possible to see biological details more clearly under a microscope. It is possible to find out how much better the images of biological samples are by using the latest Plan achromat objectives and Laowa 2.5–5X lense with a Nikon D5600 DSLR camera system [14, 15]. These three tools, namely Helicon Focus working in tandem with Zerene Stacker and Adobe Photoshop, enable us to deliver optimized depth of field management and spatial coverage workflows. Scientists use these developed procedures to acquire high-resolution imaging methods for achieving precise documentation in their research [16, 17]. The new imaging methods allow for coordinated improvements in biological imaging methods and let researchers do a variety of studies in taxonomy, histology and microbiology.

MATERIALS AND METHODS

Imaging Setup

To image plant specimens, a high-resolution photomicrography system was created. For high-fidelity depth-of-field imaging, as well as accurate microscopic visualization, two separate imaging setups were utilized. The first set-up used a Nikon D5600 DSLR camera with a Laowa 25 mm f/2.8 ultra-macro lens on a NISI 180 mm manual focus rail. It was paired with an automated MJKZZ Qool Rail 250 [18] motorized system that could accurately position the camera to within microns for 3D imaging. This arrangement allowed for obtaining images at different depths of field. The second setup used an Amscope T380C trinocular microscope with Plan Achromat objectives (4x, 10x, and 20x) for imaging with fewer chromatic aberrations and a low aperture for a flat-field image. An adapter was used to mount a DSLR camera on the trinocular head, and a mechanical shutter release cable was used to prevent vibrations during exposure. In both cases, the Godox TT520II flash unit with the Radiant diffuser was used to provide uniform illumination for both setups, reducing glare and ensuring an even light distribution. Plant samples were sectioned using a semi-automated microtome to allow for precise, uniform slicing to achieve the best imaging possible. The specimens were flexibly stabilized in a customized sample holder to obtain consistent positioning, thereby reducing the incidence of imaging artifacts. This made it possible to perform high-resolution imaging with improved depth-of-field adjustments, which was essential for structural analysis with high specificity.

Software for Focus Stacking and Image Stitching

Focus stacking was performed using Helicon Focus and Zerene Stacker, which consolidated multiple focal planes into a single composite image with extended depth of field. We captured overlapping image tiles for large specimens beyond the field of view by laterally repositioning the NISI manual rail. These tiles were stitched into a seamless panorama using Microsoft Image Composite Editor, employing feature-

based alignment to maintain spatial and resolution accuracy. Post-Processing final adjustments were applied to stacked and stitched images using Adobe Lightroom Classic and Adobe Photoshop. Global corrections included colour balance calibration, exposure normalization, and distortion control. Localized refinements addressed residual debris, uneven illumination, and edge artifacts. Sharpening was applied selectively using high-pass filters to enhance fine structural details without introducing noise.

Plant Specimen Preparation Method

For detailed photomicrography of plant specimens, chemical solutions were used to make the structures clear and the specimens well preserved. During their initial distilled-water rinse, and the surface debris from the specimens was removed. It was a chemical solution with 50% ethanol, 5% glacial acetic acid, 10% formalin (40% formaldehyde solution), and 35% distilled water that was used to fix the plant specimens. Long-term structural preservation and detailed preservation were both achieved by the fixative solution, which helped to protect proteins and other parts of cells. The specimens underwent a gradual ethanol dehydrating process, starting with 30% ethanol, then moving up to 50%, 70%, 90%, and finally concluding with 100% ethanol solutions for a duration of 10 minutes in each step to maintain tissue integrity. The dried specimens were treated with 100% xylene for 15 to 20 minutes to make the tissues clear and help them absorb the embedding medium better. The samples were embedded in paraffin wax, which was heated to 60°C. Then, they were cut into thin slices (11 µm) using a microtome, which were then used for microscopic examination.

Insect Specimen Preparation Method

Certain methods were applied to prepare insect specimens, aiming to maintain the integrity of the morphology while allowing systematic assessment. Cleaning of the surfaces started with careful, delicate manipulation to remove debris using soft bristle brushes and fine forceps. Smaller specimens were soaked in a low-foaming detergent solution and rinsed with distilled water. To protect the structural integrity, cleaned specimens were stored in 70% ethanol. The specimens were then transferred to successively higher grades of ethanol (from 70% to 100%) for 10–15 min at each concentration to minimize distortion.

Microscope and Focus Rail

The study used a high-quality affected microscope (AmScope T380C) with plan achromat objectives to get clear flat-field images with less chromatic aberration. The objectives provided a uniform focus over the entire field of view, enabling detailed viewing of plant specimens. Plan achromats are great for imaging tasks during in-depth anatomical and morphological analysis because of their high resolving power and almost no chromatic aberration. During the microscopy process, the specimens were looked at twice through 4x, 10x, and 20x, objectives, which gave images with varying levels of specificity. The combination of the AmScope T380C microscope and the Plan Achromat objectives allowed clear, high-resolution images suitable for photomicrographs. A motorized MJKZZ Qool Rail system was used as the focal depth image-capturing device for the insect photography project. This system required only one motor to drive and possess precise control, which enabled fine movement across the focal plane. The depth of field was measured, the specimen total depth was calculated, and the properties of the chosen objective lens were used to figure out the step size. The fine step size between 1–2 µm was used at 20x objective, while for images taken at 4x and 10x, the step size was much larger, between 5–10 µm. We calculated the number of steps were calculated using the following formula:

Number of Steps = Step Size Total / Depth of Field (DOF)

Focus Stacking for High-Resolution Imaging

Focus stacking, a vital high-resolution microscopy technique, generates combined images from separate photos which display all three-dimensional features in distinct detail. The method involves taking several images of the same specimen at different depths and combining them into a single composite image by using the points of highest focus in each photo. This technique becomes crucial when working with 4x, 10x, and 20x objectives because their small depth of field renders entire fine structural elements out of focus in single images. The first step is determining the overall specimen height, then selecting the appropriate step distance as a function of the depth of field and the selected objective magnification. The machine captures sequential photographs across the designated focal plane region. Software used for stacking includes Zerene Stacker, Helicon Focus, and Photoshop, which stack the photos once they have been realigned to correct any positioning error. A custom software application combines the best-focused regions from each image to create a single final composite image that has an extended depth of field to reveal details obscured by shallow depth of field. Focus stacking is incredibly useful in microscopy and macro photography, as it allows to create sharp visualizations of three-dimensional objects throughout their entire focal depth.

Focus Stitching for Expansive High-Resolution Imaging

Focus stacking is a powerful technique that allows to capture a large, high-resolution image of specimens that are too large to fit within either a microscope or a camera image. The technique is based on taking overlapping images of the sample and stitching them into a larger mosaic using a dedicated software.

It starts by taking a series of overlapping photographs in a grid, using maximum focus on every focus plane. Each frame may be used with focus stacking techniques to improve sharpness and depth of field. The acquired images are imported into focus stitching software such as Image Composite Editor or Photoshop. This transaction supports automatic extensions, whereby the shift rate when the common reference points in line sections are matched will oversee the reveal and bring-in clusters. This technique generates a single high-resolution image of the entire specimen, unmasking details that would be impossible to investigate using the traditional approach in the same way due to size constraints. It is especially helpful for taking pictures of big or complicated samples so that small but detailed structural features can be studied all over the sample. This is because it gives both high-resolution pictures and a wider field of view.

Focus Stacking Combined with Focus Stitching

Focus stacking and focus stitching have changed high-resolution microscopy by making it possible to take detailed pictures of complicated specimens. Finally, focus stacking, as the name implies, is from multiple images at different focal planes and merging them into a single image showing greater sharpness and depth of field. This method is especially useful for three-dimensional specimens with low depth of focus due to high magnification. In contrast, focus stacking consists of taking overlapping images of a larger area of the specimen in order to obtain a composite, wide-scale field of view. When used in combination, these techniques enhance both the depth of focus and the field of view, enabling sharp, high-resolution imaging of large specimen structures. In botany, for instance, focus stacking displays detailed cell layers in plant tissues, while focus stitching allows entire plant sections to be captured in high definition. Likewise, in the realm of entomology, these approaches visualize the overall body plan of an insect as well as its inner structures.

In practice, multiple images are captured in a grid fashion, with each image focused at different depth and region of the specimen. Specialized software tools, like Zerene Stacker, Image Composite Editor and

Photoshop, are used for, aligning them first by focus stacking and then merging them by focus stitching. The resulting method produces a seamless, perfectly focused image with high resolution over a broad area, making it an invaluable technique for fields such as botany, histology, or entomology, wherein intricate subjects need to be analysed.

Image Processing Workflow from RAW to Final Output

RAW files, used to store uncompressed image data, are perfect in high-resolution microscopy cases as they keep the detail level and allow full, uneconomical editing. It starts with Lightroom, where initial adjustments are made in exposure, contrast, and white balance. It cuts down on luminance noise by up to 80%, which also cuts down on high-frequency noise. It also fixes optical distortions caused by the microscope setup so that the images are lined up correctly.Smart objects export images for non-destructive changes in Photoshop. The focus stacking is done with Photoshop's Auto-Align and Auto-Blend Layers tools, which stack on top of one another photos taken at different focal planes into a single picture with an improved depth of field. The last thing to do is adjusting contrast via curves and levels, correcting colour accuracy, and applying sharpening via a high pass filter (1-2 pixel radius) blended in overlay or soft light mode. A bit of subtle dodging and burning brings the depth and vibrance, and saturation levels are slightly raised in order to bring out the natural colours without oversaturation.

RESULTS

Based on these considerations, the study concludes that the aperture from which an image looks sharpest varies depending on the magnification level. At lower magnifications (2.5x–3x), wider apertures are fine, using f/2.8–f/4 apertures. At intermediate magnification (3x–3), should be used apertures ranging from f/4 to f/5.6 (5x), as this setting balances both depth of field and resolution effectively. At higher magnifications (4x–5x), opted for narrower aperture, with f/5.6–f/8 serving as the optimal range, reduces diffraction blur and maintains sharpness, including both contrast and edge sharpness. Overall, the f/4-f/5.6 aperture range provides the best overall results at most magnification levels, yielding sharp, well-illuminated, and detailed photomicrographs (Figure 1).

It was found that the best step size for the MJKZZ Automated Qool Focus Rail was inversely related to magnification. For better depth accuracy, smaller steps were needed at higher magnifications (Figure 2). At lower magnifications (1x–3x), step sizes of 100–25 µm were sufficient to preserve depth of field. At medium to high magnifications (4x–5x), steps as small as 10–5 µm made things a lot clearer. At very high magnifications (10x–20x), steps as small as 2.5–1 µm were needed to see fine structural details without losing definition.

An AmScope T380C microscope imaged a transverse section of a wheat stem at 100x, demonstrating the power of combined focus stacking and stitching. 130 individual frames were focus-stacked for an extended depth of field, while 8 overlapping images were stitched for an expanded field of view. This combined output showed the stems internal cellular architecture with a high level of detail, which made it possible to analyse the body in great detail (Figure 3).

Focus stacking at 4x magnification demonstrates it applicability to the field of entomology. Single-exposed images couldn't show these fine morphological details, such as the setae, segmented antennae, and compound eye textures (Figure 4). However, a 731-frame stack of *Phaonia rufiventris* made with a manual NiSi focus rail could show all of these details. A 76-frame stack of *Xylotrechus quadripes* (coffee white stem borer) showed exoskeletal features like patterns and segmentation on antennae that could be used to identify the taxon. The images were as sharp as those taken with tripods at high frames, even

though they had a lot fewer input (Figure 5). These results confirm that focus stacking is a powerful tool for reducing out-of-focus artifacts and achieving a high resolution across different biological samples.

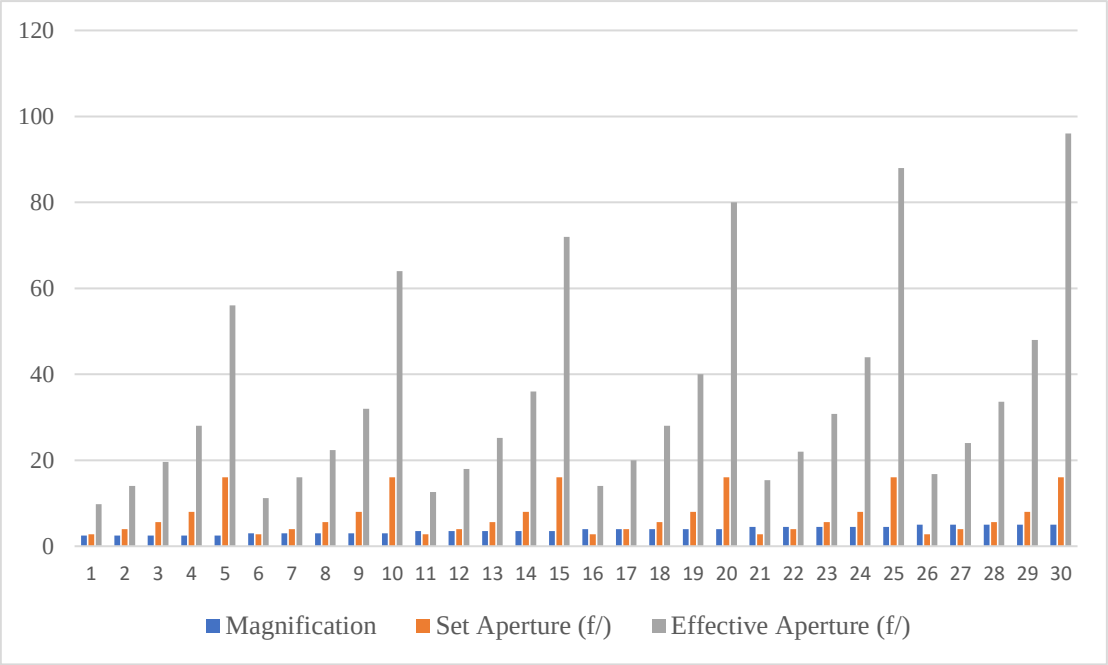

Figure 1: Effective Aperture Values for Various Set Apertures at Different Magnifications.

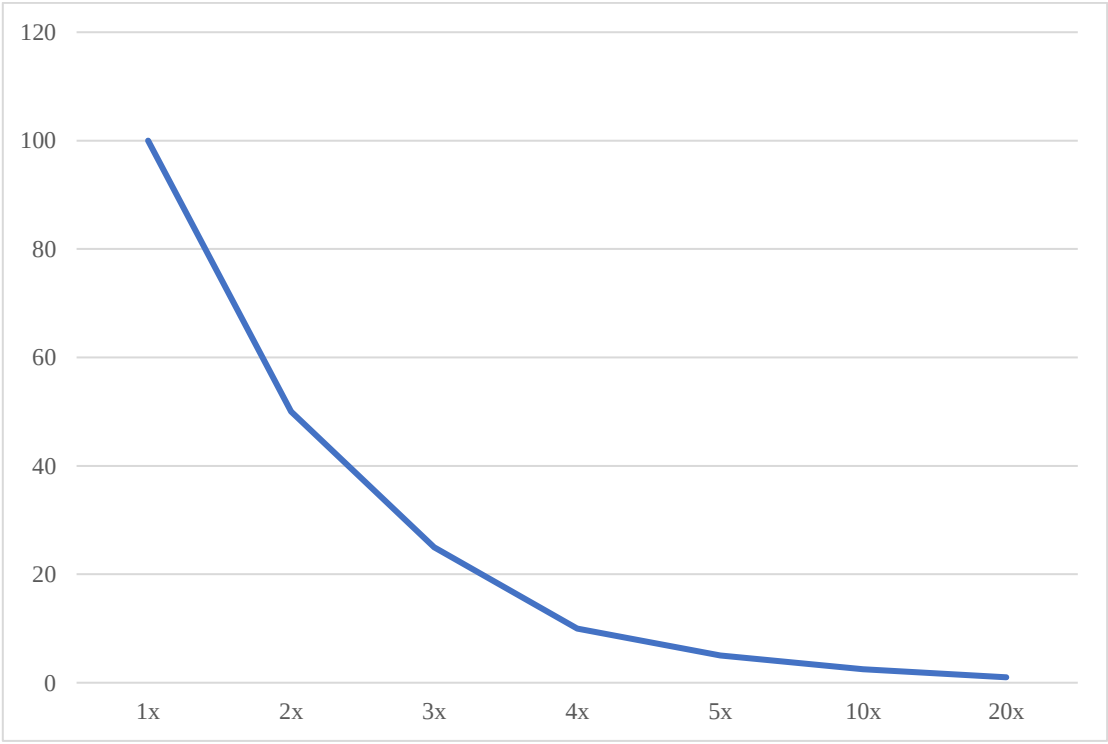

Figure-2 Optimized Step Size for MJKZZ Automated Qool Focus Rail at Different Magnifications.

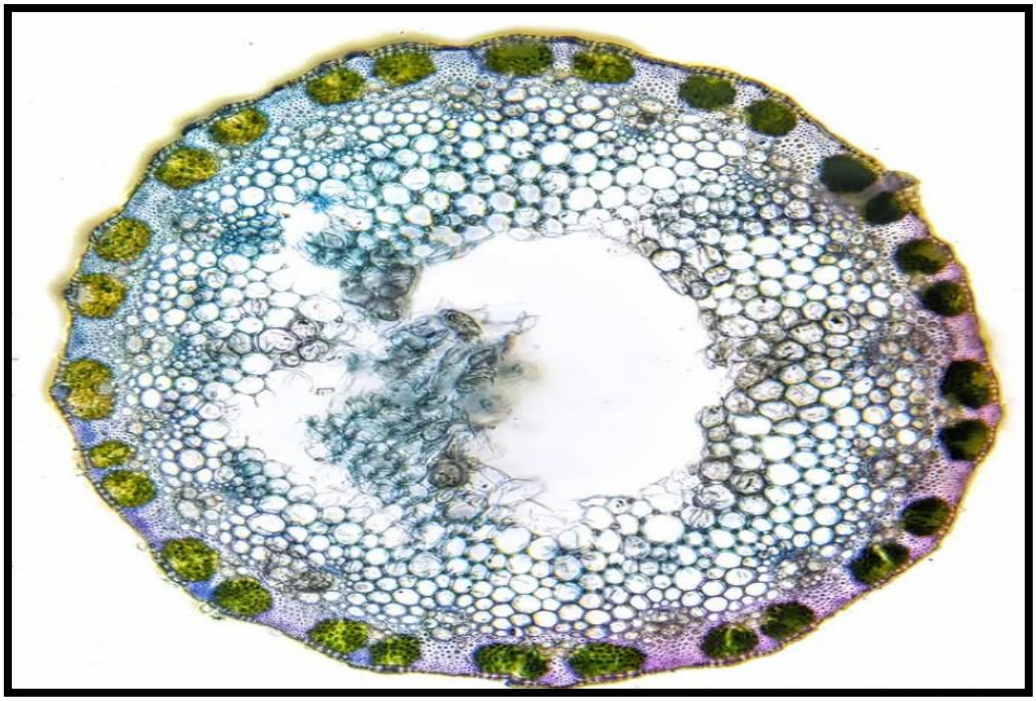

Figure-3 Wheat stem transverse section at 100X, captured with Amscope T380C. Focus stacked (130 images) and stitched (8 frames) using MS Composite Editor.

Figure-4 High-Resolution 4x Magnification Focus-Stacked Image of *Phaonia rufiventris* Fly Using 731 Frames Captured with a Manual NiSi Focus Rail for Enhanced Depth and Clarity.

Figure-5 High-Resolution 4x Magnification Focus-Stacked Image of *Xylotrechus quadripes* Using 76 Frames for Enhanced Clarity and Morphological Detail.

DISCUSSION

In the realm of high-resolution photomicrography, the integration of focus stacking and stitching techniques has become essential for producing images with enhanced depth of field and expanded fields of view. These methods involve capturing multiple images at varying focal depths and positions, which are then meticulously combined using specialized software to create a single, comprehensive image. This approach not only overcomes the limitations of shallow field depth inherent in high-magnification microscopy but also ensures that the final images maintain high resolution and clarity across the entire specimen. The application of these advanced imaging techniques is crucial for detailed analysis in scientific research, as they provide a more complete and accurate representation of microscopic structures.

FUTURE ASPECTS

There is a balance between resolving power and focal plane distance in high-resolution microscopy. This means that adjusting focal strains between magnification and depth of field is hard to do without AI-driven automation or real-time colour correction. It would help automate workflows, promote consistency across large data sets, and improve the overall image quality. Improved Mitutoyo apochromatic objectives, or other lens design advances, may also lead to higher resolving power and even lower chromatic aberrations. By using machine learning, image processing software could use algorithms that are good at recognizing features, like the structures of cells, and require less work from humans when it comes to stacking and stitching. The advances will help enhance specimen documentation accuracy and spur innovation in biological imaging and diverse disciplines where high-fidelity, high-precision data are needed.

CONCLUSION

These features, together with the productive combination of high-quality optical devices (e.g., Mitutoyo objectives) and advanced image processing, like Lightroom and Photoshop, provide a powerful workflow for high-resolution microscopy. RAW shooting with in-depth post-processing guarantees the greatest clarity, truest colour, and extended depth of field. Scientists in fields like Botany, Zoology and Microbiology find this method useful because it lets them see three-dimensional structures. Methods like focus stacking and stitching make this possible. Scientists can use them to create scientifically accurate and visually exact images for research, diagnostics, and educational purposes.

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