Combined application of extended depth of field imaging, image stitching and polarized microscopy techniques in identification of *Spatholobus suberectus*  

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**Abstract**  

**Objective:** As traditional techniques for microscopic identification of Chinese medicines currently lack objective and high-quality reference images, here we developed a systemic procedure to be used in microscopic identification of Chinese medicines, which would lead to more objective, effective and accurate identification process.  

**Methods:** *Spatholobus Caulis* (Jixueteng in Chinese) was used as the specimen in the development of such procedure. Jixueteng samples were microscopically examined in bright- and dark-field microscopy. Microscopic images were obtained by regular, EDF, and image stitching techniques.  

**Results:** The microscopic images of the characteristics in pulverized Jixueteng were captured, thanks to EDF imaging and image stitching techniques which allowed the detailed and full sighting of each characteristic to be obtained simultaneously. Different layers in anatomical transverse section, including cork, phelloderm, cortex, phloem, cambium, xylem and pith, were distinctively observed. Moreover, by comparing images of bright- and dark-field microscopy, birefringent and non-birefringent components could readily be distinguished.  

**Conclusion:** With application of the developed procedure, high-definition, panoramic and microscopic images were acquired, which could be used as the reference images for microscopic identification of Chinese medicines.

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1. Introduction  

Microscopic identification of Chinese medicines refers to microscopic examination of the anatomical sections, tissue and cellular components of various forms of Chinese herbal medicines, such as crude drugs, decoction slices and preparations, etc (ChP, 2020). The technique was firstly adopted in Chinese Pharmacopoeia as one of the identification techniques in 1977 edition (ChP, 1977), and quickly became one of the most critical methods for authentication and quality assessment of Chinese medicines, thanks to its direct, rapid, economical and environment-friendly advantages.  

In the past, due to the limitations of microscopes and imaging devices, most of the microscopic images obtained were of low quality. The reference images used in related official standards and academic articles were mostly one-shot images (Li, & Qian, 2009; Zhao, & Chen, 2016; Zhang, Xiao, & Liu, 2000) and/or sketch drawing of microscopic characteristics (Xu, 1986; Lou, 2003; Xu, & Xu, 1997), which could be misleading to the readers, as the shape and color of the images differed drastically from the reality.  

Nowadays, following the development of more cutting-edge equipment and sophisticated imaging techniques, premium quality microscopic images could be captured. Extended depth of field (EDF) imaging technique allows multi-point focusing which resolves blurry image issue due to rough surface of the specimen. Image stitching technique enables procurement of a large, full-view microscopic image of anatomical sections, which is characteristic for each kind of herbal medicines. Polarized microscopy allows distinguishing of birefringent and non-birefringent tissue components in the specimen, which is useful for both identification and authentication processes (Jin, 2016; Michael, 2014; Sutcharitchan et al., 2018; Shabnum et al., 2019).
Spatholobi Caulis (Jixueteng in Chinese) refers to the dried stem of Spatholobus suberectus Dunn. The crude drugs of this medicine are characterized by red-brown phloem tissues and xylem alternately arranged into concentric ellipse-shaped ring or eccentric semicircular rings. Also, several microscopic characteristics, such as prismatic calcium oxalate crystals, vessels with bordered pits, and sclereids, could easily be observed in pulverized crude drugs and anatomical cross sections of Jixueteng (Chp. 2020).

Therefore, in this study, Jixueteng was used as the representative specimen of Chinese medicines. Bright- and dark-field microscopy were performed on pulverized Jixueteng. Microscopic images were obtained by regular, EDF, and image stitching techniques. The resulting images were examined and compared in this article.

2. Materials and methods

2.1. Plant materials

Eleven batches of Spatholobi Caulis (Jixueteng), as shown in Table 1, were used in this study. All of the samples were authenticated by Prof. Ya-jun Cui. The voucher specimens of the samples were deposited at Department of Pharmacognosy, Shanghai University of Traditional Chinese Medicine, China.

2.2. Instruments

Microscopy was performed on Leica DM6 B and Nikon 80I microscopes. Microscopic images were obtained by Leica DMC6200 and Nikon DS-R1I digital imaging systems.

2.3. Methods

2.3.1. Application of extended depth of field (EDF) imaging technique

The intact crude drugs of Jixueteng were pulverized into fine powder and passed through #80 sieve. Chloral hydrate (75%) solution was used as clearing reagent in microscopic examination of crude drug powder, and glycerin was added as the mounting medium.

The bark of Jixueteng was scraped from the crude drugs into a test tube. Five percent sodium hydroxide solution was added until the samples were completely submerged. The test tube was sonicated at 53 Hz and 60 °C for 30 min, then put aside for 2 h. The mixture was drawn out of the test tube and dropped on to a clean microscopic slide. After putting over a cover slit, the samples were rinsed with water, and glycerin was added as the mounting medium.

Important microscopic characteristics of jixueteng include prismatic calcium oxalate crystals, vessels with bordered pits, and sclereids. For each characteristic marker, one-shot microscopic images were captured with regular imaging technique at certain different focus points, whereas extended depth of field (EDF) technique was performed by setting the start point, end point, and the number of shots. EDF images were automatically generated by the imaging system. The resulting images were compared with one-shot images of the same characteristics.

2.3.2. Application of image stitching technique

(i) Application in examination of microscopic characteristics

Pulverized Jixueteng was prepared as described in 2.3.1. Images of the microscopic characteristics of jixueteng were captured at 10× and 40× magnification. Images at 40× magnification were merged into one large image by imaging stitching technique. The resulting images by the two different methods were compared.

(ii) Application in examination of anatomical sections

Pulverized Jixueteng was prepared as described in 2.3.1. Bright-field microscopy was performed on dyed cross section of jixueteng. Microscopic images were captured at 4× magnification, and merged into one large image by imaging stitching technique of the imaging system.

2.3.3. Application of microscopic imaging techniques under polarized light

Pulverized Jixueteng was prepared as described in 2.3.1. Dark-field polarized microscopy was performed on the dyed cross section and pulverized jixueteng. Microscopic images were captured by regular and EDF imaging techniques. Some were merged into one large image by imaging stitching technique. Bright-field microscopy was performed in the same position of each characteristics, and the images obtained were compared with those from dark-field microscopy.

3. Results

3.1. Application of extended depth of field (EDF) imaging technique

Owing to multiple focus points, extended depth of field (EDF) imaging technique was able to capture more detailed characteristics than the regular imaging technique. As shown in Fig. 1 (right), two of the prismatic calcium oxalate crystals in pulverized jixueteng were captured in focus in the same frame by EDF technique. In the images captured by regular technique (Fig. 1, left) one of the crystals was defocused.

For characteristic markers with rough surface, EDF imaging technique enabled obtaining of high-quality microscopic images in which important details on different focal planes could be obtained. As shown in Fig. 2 (right), bordered pits on the vessels of jixueteng captured by EDF technique were all in focus, whereas those in the images captured by regular technique were partly defocused (Fig. 2, left). The pits and stripes of sclereids and sclereid clusters were acquired clearly by EDF imaging technique (Fig. 3, right), while partly defocused in the images by regular imaging techniques (Fig. 3, left).
3.2. Image stitching technique

3.2.1. Application in examination of microscopic characteristics

As shown in Fig. 4 (1), the image captured by regular imaging technique at 10× magnification showed complete view of the crystal sheath fibers from which the length could be measured. However, the detailed characteristics, such as the stripes and pits, could not be captured at 10× magnification but became quite distinct at 40× magnification, as shown in Fig. 4 (2). The image stitching technique allowed merging multiple images of 40× magnification into one large panoramic image, in which the details could be observed while maintaining the full view of the object, as shown in Fig. 4 (3).

3.2.2. Application in examination of anatomical sections

Fig. 5 (1) and (2) are close-up images and sketch drawing of the anatomical transverse section of Spatholobi Caulis (Jixueteng) currently used in An Illustrated Handbook on Microscopic Identification of Chinese Crude Drugs for Chinese Pharmacopoeia. Image stitching technique combined the advantages of regular one-shot imaging technique and sketch drawing, allowing anatomical details to be clearly obtained in a full-view image, as shown in Fig. 5 (3).

3.3. Application of microscopic imaging techniques under polarized light

Birefringence is one of the important properties used in distinguishing one component from another under polarized dark-field microscopy. Common birefringent plant components include xylem, sclereids, calcium oxalate crystals, vessels, etc. (Wang et al., 2016). As shown in Fig. 6, a large high-definition microscopic image of anatomical cross section of Spatholobi Caulis (Jixueteng) was obtained by image stitching technique under polarized light, allowing birefringent and non-birefringent components to be distinguished, and the birefringent characteristics in anatomical cross section to be readily observed.
Fig. 7 showed close-up images of anatomical cross section of Jixueteng obtained under bright- and dark-field microscopy in comparison. Under dark-field microscopy, birefringent components, including xylem and sclereids, appeared clear and bright, while other components were screened out by the polarized-light. Figs. 8–11 showed comparison of microscopic characteristics of Jixueteng at cortex, phloem, xylem, and pith layer, respectively. Xylem, sclereids, calcium oxalate crystals were clearly observed under polarized light while swallowed by other components in bright-field microscopy.

4. Discussion

Microscopic identification is considered as a useful traditional method for identification and authentication of Chinese medicines. Although it possesses direct, rapid, economical, and environment-friendly advantages, the effectiveness of the method is often challenged by other novel methods which involve high-technology equipment.

In this study, this traditional method of identification was combined with sophisticated equipment and techniques for the efficient identification of Chinese herbal medicines taking Spatholobi Caulis (jixueteng) as an example. A systematic procedure, involving the application of both bright- and dark-field microscopy, extended depth of field (EDF) imaging, and image stitching techniques, was developed. Through the application of aforementioned techniques, high-definition, panoramic, microscopic images were obtained, leading to more effective and time-saving process of microscopic identification.

Spatholobi Caulis (Jixueteng) was used as the representative specimen in this study because of its distinctive macro-, and microscopic characteristics. Using the developed procedure, different layers in anatomical transverse section, including cork, phelloderm, cortex, phloem, cambium, xylem, and pith, were clearly observed. The microscopic characteristics in pulverized Jixueteng were captured in detail. For characteristics with rough surface, EDF imaging technique enabled obtaining of premium quality microscopic images in which important details on different focal planes were all achieved.

The image stitching technique allowed merging multiple images at higher magnification into one large panoramic image, in which the details could be observed while maintaining the full view of the object. Under polarized dark-field microscopy, birefringent characteristics became very distinctive. By comparison of bright-field with dark-field images, the birefringent and non-birefringent characteristics were readily distinguished.
5. Conclusion

Through combination of EDF and image stitching techniques with bright- and dark-field microscopy, high-definition, panoramic, microscopic images could be obtained. The microscopic characteristics of Chinese medicines could readily be distinguished from non-characteristic components. This study served as a demonstration of the application of a novel approach in microscopic identification of Chinese medicines, by which the images obtained might be suitable as the reference images for microscopic identification in official standards.
Fig. 6. Anatomical cross section of Spatholobi Caulis (Jixueteng) under polarized light.

Fig. 7. Comparison of anatomical cross section of Spatholobi Caulis (Jixueteng) under bright-field and dark-field (polarized-light) microscopy (1. Cork; 2. Phelloderm; 3. Cortex; 4. Phloem; 5. Cambium; 6. Xylem; 7. Pith).
Fig. 8. Comparison of microscopic characteristics of Spatholobi Caulis (Jixueteng) at cortex layer under bright-field and dark-field (polarized-light) microscopy (1. Prismatic calcium oxalate crystal; 2. Sclereid).

Fig. 9. Comparison of microscopic characteristics of Spatholobi Caulis (Jixueteng) at phloem layer under bright-field and dark-field (polarized-light) microscopy (1. Sclereid; 2. Phloem fiber; 3. Prismatic calcium oxalate crystal; 4. Secretory cell).

Fig. 10. Comparison of microscopic characteristics of Spatholobi Caulis (Jixueteng) at xylem layer under bright-field and dark-field (polarized-light) microscopy (1. Xylem fiber; 2. Prismatic calcium oxalate crystal; 3. Vessel; 4. Xylem ray).

Fig. 11. Comparison of microscopic characteristics of Spatholobi Caulis (Jixueteng) at pith under bright-field and dark-field (polarized-light) microscopy (1. Secretory cell; 2. Prismatic calcium oxalate crystal).
Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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