Image analysis of transmission electron micrographs of citrus greening bacterium and chilli veinal mottle virus

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ABSTRACT: A TEM imaging result would be a black and white film negative, which needs to be processed. A workflow to scan and analyze such negatives was developed using a Personal Computer with a top illuminated scanner. The 9x6.5 cm TEM negatives and 35 mm TEM negatives were scanned at 1200 dpi and were saved as TIFF, jpg, raw and png files. Using ImageJ the samples were false colored to bring out features using Lookup Tables based on their image densities. Using this method color or black and white negative (CAT, X-ray, and MRI) can be digitized and analyzed. Even though recent TEM and SEM have digital printout facilities as the bulk of the existing imaging systems in India use film, this method of scanning and analysis we have described will help in digitizing archival films and help in the digital storage, analysis and transmission and will be of use in any lab using scans.

Keywords: Density shading, ImagJ, Scanning, TEM micrographs

INTRODUCTION

The transmission Electron Microscope (TEM) enables visualizing a single virus particle. But the TEM imaging result would be invariably a black and white film negative as electrons lack a color spectral characteristic. At the first exposure of the film to the electron source a small number of atoms of silver would get created but this image would be invisible to the naked eye as it is a latent image. This latent image would be amplified or developed by using a chemical process to form a visible image that would be black in the sites where the electron beam had excited it. Further the remaining silver halide crystals that were not exposed to the light would be removed by a process called as fixation. This process needs to be repeated to get a positive from the negative which is the final print. The procedures for processing, printing and enlarging of such films have become extinct due to the development of fast modular color photo shops. As the TEM negatives are larger than 35 mm film negatives they don’t fit in these modern machines, leading to an inability to get easy printouts at the desired size. More over highly skilled labor and time is required for photo processing which are not available now. In the past 25 years, the original NIH Image program had meta morphed into the ImageJ2 software (Caroline et al., 2012). ImageJ had been the pioneer as an open tool for the analysis of scientific images (Abramoff et al., 2004). It had also given rise to similar programs like Fiji (Schindelin et al., 2012), Bio Image XD (Kankaanpää, et al., 2012) and Icy (de Chaumont et al., 2012). The utility of ImageJ in solving common microscopic and ultra-structural image analysis tasks had been reviewed (Papadopulo et al., 2007, Collins, 2007). ImageJ was reported to be sufficiently flexible to be adapted to the numerous requirements tasked in the laboratories like, areameasurements on selectively stained tissue components, cell counts and area measurements at single cell level, immune his to chemical antigen quantifications, and using immune electron microscopy gold particle counts. It was also reported that the near comprehensive range of import filters available in ImageJ allowed easy access to image and Meta data. The availability of a broad suite processing and analysis routine, and enthusiastic support from a friendly mailing list were noted to be invaluable for all microscopy labs. Analysis protocols using ImageJ to provide quantitative assessment of microscopy data, through analysis of objects in a fluorescent image, for extracting information from two color tissue images collected by bright field microscopy and histological staining and fluorescence analysis are available (Sean, 2013, Jensen, 2013). The open source advantage of ImageJ had resulted in the development of specific macros and plugins suited for specific tasks like a plugin (Jensen, 2013) for accurate evaluation of disease phenotypes defined as the rate of host colonization by the pathogenic microbe by calculated values which determined the disease index for a specific treatment and...
thereby provided a basis for the unbiased identification of candidate host genes required for full resistance to fungal infections (Abd-El-Haleem, 2012). AG Scan (Cathelin et al., 2007) another plugin based on the Image J library had been used for micro array analysis. Leaf J was used to rapidly measure petiole length and leaf blade parameters of the model plant Arabidopsis thaliana to quantify the leaf area accurately (Maloof et al., 2013).

MATERIALS AND METHODS

To scan the TEM negatives, Citrus plants infected by Greening Bacterium (CGB) (Graca, 1991) were used. The samples from CGB infected bud wood were collected from declining orchards and were processed for resin embedding and were cut from blocks using the Reichert Ultramicrotome® with glass knives. 50 nm sections were made, dried, incubated with Uranyl Acetate stain. The sections were viewed in JEOL 100 TEM and were photographed in 9x6.5 cm TEM Kodak Ektachrome® film negatives. For scanning from 35 mm TEM negatives, leaf tips of a Chilli veinal mottle virus Potyvirus infecting Chilli were made, dried, incubated with uranyl acetate stain and were viewed in JEOL 100 TEM. The images were photographed on 35 mm film using the camera attached to the side port of the TEM. Both the TEM film and the 35 mm film were processed to obtain the negatives.

The PC system used to scan was a 4 core i7 processor, with 32GB RAM, with an nVidia 640 Ti video card running windows 8 64 bit software, with 6 TB hard discs. The film scanner Canon Scan 9000F with Top illumination slide scanning capability (Fig.1.) was controlled by 64 bit Vue Scan software (http://www.hamrick.com). The 9 x 6.5 cm TEM negatives were scanned at 1200 dpi and were saved as TIFF, jpg, raw and png files (Fig. 2.) Using ImageJ2 version 1.49G (http://imagej.net/ImageJ2) the tiff image were opened and the following operations were carried out on them. The scanned TEM negative was inverted to get the positive image, the TEM negative was red, green and blue tinged using respective, 0-255 value LUT’s, the TEM negative contrast was enhanced by local enhancement. Look up tables (LUT) which are basically tables of
equivalents which converted the brightness and darkness present in an image into numbers were used to pseudo colour the scans by using a 8-bit grey scale system where minimum LUT value was set to zero, and maximum LUT to 255, with all of the other gradations of intensity given values between 0 to 255. The LUT’s were created in a text editor and used. The following LUT’s were applied to the scanned TEM negative, Fire, Green to red, 16 colours, 20 colours, 32 colours, Spectrum, 50% cut-off green to red, Rainbow, ICA Profile. MEASURE toolbox was used to calibrate the screen size with real world measurements. Similar to the operations on the TEM negatives, the 35 mm film negatives were also scanned (Fig. 19). The scanned 35 mm TEM negative was inverted to get the positive image, and the following LUT’s were applied 20 colours LUT, Rainbow LUT Iso contour LUT, Phase LUT, Topography LUT. Further the following Image processing routines were done on the 35 mm scanned images. (a) The background clean plugin with LUT inverted green to red, (b) Background clean plugin with LUT 20 colours, (c) Find edges plugin with LUT 32 colours, (d) Background clean plugin followed by sharpen plugin, followed by enhance contrast plugin with LUT amber, (e) Sharpen plugin applied twice with spectrum LUT as described (http://rsbweb.nih.gov/ij/docs/user-guide.pdf)

RESULTS AND DISCUSSIONS

Scanning negatives with top illumination gave good results when printed. Inverting and printing the scanned Images gave printouts whose print quality were adequate with sufficient contrast without the use of a black room and chemicals, further as the scanning was at 1200 dpi, the images could be magnified without pixilation (Figs. 2, 20, Plate. 4.). Tingeing the TEM negatives (Figs. 7-9) did not increase the information derived from the image as it merely replaced the black colour with the respective tinge. Local contrast enhancement to increase the appearance of large numbers of light to dark transitions increased the appearance of small scaled edges and gave good imaging
results so that CGB could be visualized clearly (Fig. 10). All the LUT’s used (Fig. 8-16, 21, 28-30) differentiated the black and white image based on its density and Pseudo colouring made the identification clearer. Fire LUT, (Fig.8) gave exceptional differentiation of the CGB samples as blue spots with the background yellow in colour. Among the other LUT’s tried, Green to red LUT, 20 colours LUT, Rainbow LUT and ICA profile LUT differentiated the background and the sample (Fig. 9, 11, 15, 16).

Using the MEASZURE (http://www.thing.com/Measure.asp) toolbox (Fig.17), the dimensions of the organelles in the scan were measured (Fig.18). Cleaning the background by rolling circle method followed by application of LUT’s gave images with a higher level of clarity (Fig. 23, 24). Find edges plugin with LUT of 32 colours enabled the visualization of the virus particles clearly (Fig. 25). Use of combined plugins like Background cleaned, Contrast enhanced with Amber LUT gave extremely differentiated images (Fig. 26). Sharpen plugin applied twice with spectrum plugin also gave good results (Fig. 27). The scanned 35 mm TEM image was reduced in size to a square of 1 and 3 cm2, it was also magnified to 4.5,6,12,15,26,92 cm2 size. The program MEASURE was used to measure the cell constituents by calibrating the magnification values with screen pixels to nanometer and the length of the cells, organelles and Citrus Greening Bacterium were measured (Fig. 17). This program was also used to show a screen ruler to determine the magnification (Fig. 18). Due to the 1200 dpi scan, the TEM images could be magnified without any pixilation (Plate 4).

While recent TEM and SEM have digital printout, in reality the bulk of the imaging systems in India use film, the method of scanning and analysis we have described will help in digitizing archival films and help in the digital storage and transmission and will be of use in any lab using scans as any color or black and white negative (CAT, X-ray, and MRI) can be digitized and analyzed with its attendant benefits. The addition of color information increases the information content of the images and will be benefit in diagnosis.

REFERENCES


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