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Desktop scanning electron microscopy in plant-insect interactions research: A fast and effective way to capture electron micrographs with minimal sample preparation

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3 **Desktop scanning electron microscopy in plant-insect interactions research: A fast and**
4 **effective way to capture electron micrographs with minimal sample preparation**
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Abstract:

The ability to visualize cell and tissue morphology at a high magnification using scanning electron microscopy (SEM) has revolutionized plant sciences research. In plant-insect interactions studies, SEM based imaging has been of immense assistance to understand plant surface morphology including trichomes (plant hairs; physical defense structures against herbivores (Kaur and Kariyat, 2020a, 2020b; Watts and Kariyat, 2021), spines, waxes, and insect morphological characteristics such as mouth parts, antennae, and legs, that they interact with. While SEM provides finer details of samples, and the imaging process is simpler now with advanced image acquisition and processing, sample preparation methodology has lagged. The need to undergo elaborate sample preparation with cryogenic freezing, multiple alcohol washes and sputter coating makes SEM imaging expensive, time consuming, and warrants skilled professionals, making it inaccessible to majority of scientists. Here, using a desktop version of Scanning Electron Microscope (SNE- 4500 Plus Tabletop), we show that the “plug and play” method can efficiently produce SEM images with sufficient details for most morphological studies in plant-insect interactions. We used leaf trichomes of *Solanum* genus as our primary model, and oviposition by tobacco hornworm (*Manduca sexta*; Lepidoptera: Sphingidae) and fall armyworm (*Spodoptera frugiperda*; Lepidoptera: Noctuidae), and leaf surface wax imaging as additional examples to show the effectiveness of this instrument and present a detailed methodology to produce the best results with this instrument. While traditional sample preparation can still produce better resolved images with less distortion, we show that even at a higher magnification, the desktop SEM can deliver quality images. Overall, this study provides detailed methodology with a simpler “no sample preparation” technique for scanning fresh biological samples without the use of any additional chemicals and machinery.

Introduction

While we can clearly see leaves, roots, and flowers with our naked eye- plants and animals have functionally complex and morphologically diverse structures which necessitates the need of advanced microscopy techniques to comprehend microscopic structures such as tissues and cells (Caldwell and Iyer-Pascuzzi, 2019). These structures tend to have complex morphological variations, which can only be visualized by advanced and powerful microscopy (Palaiologou et al., 2020). For example, while pollen is visible, individual pollen grains typically range in size between 25-50 μm (Kelly et al., 2002) and can be studied more effectively with microscopy. Similarly, insect antennae and trichomes also range in micrometers and are difficult to observe in detail with the naked eye. Therefore, to understand the ultra-structure and to penetrate beyond the visible surface morphology, various microscopy tools are routinely employed (Mustafa et al., 2018). Light microscope is popular and commonly used for this purpose but is limited by extremely low resolution and limited specimen compatibility (Wollman et al., 2015).

Alternatively, a Scanning Electron Microscope (SEM) can acquire images ranging from magnifications of 10X to 500,000X, using secondary electrons (Zhu et al., 2009), X-rays (Kotula et al., 2003) and electron beams (Nakamura et al., 2021) that interact with the specimen while collecting the scattered radiation to produce an image (Inkson, 2016). For the past few decades, SEM has revolutionized imaging by helping to understand the microstructure of biological samples, cell lines, superconductors, micro-crystallization of nano particles, and many more (Zhang et al., 2020a). Plant-insect interactions studies utilize SEM quite often because the field routinely examines morphological traits of insects and plants and their interactions between them, and among other components of the environment (Pathan et al., 2008; McCully et al., 2009; Kaur et al., 2020). For example, plants morphological traits including trichomes, stomata, waxes, and

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3 pollen show tremendous variation like their insect counterparts including their antennae, eggs,
4 mouth parts, legs, and wings vary immensely among them (Krenn, 2009; Singh and Kumari, 2020),
5 commonly imaged using SEM.
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10 As the use of SEM has exponentially increased in last few decades, the technological
11 advances in microscopy have also improved (Sujata and Jennings, 1991; Stokes, 2003; Newbury
12 and Ritchie, 2015; Weigend et al., 2017). However, due to the lack of innovation in sample
13 preparation methodology, SEM imaging remains a time consuming and expensive process (Inkson,
14 2016; Kariyat et al., 2017). As a result of the time consuming and expensive sample preparation
15 associated with SEM prior to the actual scanning of sample, SEM is still limited to core facilities
16 across various research and educational institutions. Due to the lack of a simpler protocol,
17 microscopists follow lengthy and complex procedures of sample preparation and image processing
18 involving fixation of sample using critical buffers (e.g., glutaraldehyde) overnight, tissue drying
19 through sequential alcohol washes, critical point drying and sputter coating of sample, which not
20 only adds up cost and efforts, but can also take minimum of 6-10 hours to get samples ready to be
21 processed for imaging (Kariyat et al., 2017; Table 1). Moreover, SEM demands skilled personnel
22 and technical expertise to acquire quality images, thus limiting its use to core facilities or big-
23 budget labs with the technicians available. It is quite evident that SEM imaging warrants
24 methodology that can retain its quality in imaging, but it can also shorten the timeline.
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45 To overcome intensive and expensive methodology associated with traditional scanning
46 electron microscopy, we have been using the SNE- 4500 Plus, a tabletop scanning electron
47 microscope (Nanoimages LLC, Pleasanton, California, USA) that requires almost no sample
48 preparation before imaging. The Desktop Scanning Electron Microscope (DSEM) can capture
49 images up to 200,000X magnification, while providing precise 3-D imaging of morphology and
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3 dimensions of structures seen in the samples. The DSEM is equally capable of capturing miniature
4 plant features and ultra-structures of objects, thus providing detailed morphological
5 characterization. For instance, one of the biggest advantages of DSEM is its ability to use fresh
6 specimens (having high water content such as leaves an almost impossible task with a traditional
7 SEM), cutting back on elaborative and tedious sample preparation time. Since DSEM requires no
8 sample preparation before imaging and only takes 15 minutes for image acquisition (Table 2), it
9 allows for more throughput processing unlike traditional SEM (Table 1). Moreover, it is user-
10 friendly as it requires little or no technical expertise, and its cost effectiveness makes it possible
11 for individual labs to possibly acquire and can also serve as a learning opportunity for students
12 who can use it on a routine basis by themselves without supervision.
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26 DSEM is even more beneficial in plant-insect interactions studies, where plant and insect
27 images are routinely studied at microscopic levels (Silva et al., 2017; Watts and Kariyat, 2021).
28 Previously, we have documented that trichomes not only act as a mechanical barrier to the
29 movement and feeding of caterpillars but can also damage the peritrophic membrane of caterpillars
30 and are even present in the frass pellets of these caterpillars (Kariyat et al., 2017; 2019).
31 Furthermore, to test for subtle differences in treatments (e.g., damaged vs undamaged; inbred vs
32 outbred plants; Kariyat et al., 2017), we require fast and throughput imaging, an almost impossible
33 feat with traditional high-performance SEM. Resorting to simple light microscopy on the other
34 hand can lead to missing key details. For example, trichomes can be classified into glandular and
35 non-glandular types, based on the presence or absence of glandular top, and silverleaf nightshade
36 (*Solanum elaeagnifolium* Cav.; Solanaceae), a worldwide invasive weed, has been found to have
37 a dense mat of non-glandular stellate trichomes (Kariyat et al., 2018; Chavana et al., 2021).
38 However, using the DSEM, we recently found that in addition to non-glandular stellate trichomes,
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3 also possess a low density of glandular trichomes, almost impossible to detect through light
4 microscopy. And, in a recent study (Watts and Kariyat, 2021), we imaged 11 Solanaceae species,
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6 and leaves for each leaf surface (adaxial and abaxial) to determine the statistical significance
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8 between treatment groups (species; leaf surface)- clearly showing the need for extensive and
9
10 thorough imaging which could have only been possible with SEM needing no prior sample
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12 preparation. DSEM can also distinguish the sub-parts of these microscopic hairs. In addition,
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14 DSEM also aided us to observe epicuticular wax along with trichomes present on the leaf surface
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16 in species like *Solanum glaucescens* Zucc. We show how DSEM can acquire images of various
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18 biological samples and their morphological features with precision, without the costs or time
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20 associated with traditional SEM.
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28 **Materials and methods**

29 **Instrument specific methodology for image acquisition:**

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31 There are four major steps in imaging using DSEM. The first step ‘Pre-Sample Preparation DSEM
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33 Operation’ involves preparing the DSEM before placing the sample in the vacuum chamber. The
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35 second step ‘Sample Preparation’ includes setting up the sample stage without the use of chemicals
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37 and other machinery. The third step ‘post-sample preparation DSEM Operation’ involves the steps
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39 to be followed post sample set up in the machine. The final step ‘Image capturing and processing
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41 using ‘Nanoeye’’ involves steps to be followed to acquire SEM image post sample insertion and
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43 vacuum build up in machine.
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49 Below is the description of these major steps:

50 **A. Pre-Sample Preparation DSEM Operation**

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3 Before turning on the DSEM and computer, the vacuum pressure should range from 90-120psi on
4 the air compressor (rotary vane pump). Confirm that the vacuum pumps are functional for the
5 entire duration of imaging and that the fastened stage with/without sample is under vacuum despite
6 the fact it is turned off to avoid any debris entering the machine. Allow the vacuum to build in
7 DSEM by pressing the 'Exchange' button. A stable green light indicates that full vacuum has been
8 established into the column containing stage inside the DSEM. After the establishment of full
9 vacuum, press the 'Exchange' switch again to initiate release of vacuum from DSEM. Then, launch
10 'Nanoeye' software associated with DSEM on the computer linked to DSEM. Then, pull out the
11 motorized stage smoothly, fully open the door, loosen the stage using hex key (Figure 5A1) and
12 take out sample along with aluminum stub using the SEM mount forceps (Figure 5A2). Select the
13 'Calibration' icon on the Motor Control Panel of 'Nanoeye' software and allow the stage to come
14 to its originally assigned 3-D position.
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30 **B. Sample Preparation**

31 Any fresh biological/non-biological sample can be used for image acquisition in SNE- 4500 Plus.
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33 A general principle for sample preparation is to get an excised sample of diameter up to 80 mm
34 and thickness up to 50 mm (based on size of the stage). Then, fix the sample onto a double-sided
35 conductive carbon tape (Figure 5C) glued to an aluminum stub of suitable diameter (15mm, 25mm
36 and 40mm) depending on the size of the sample (Figure 5B).
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45 **C. Post-sample preparation in DSEM Operation**

46 The diameter and height of stub along with sample should be recorded using the machine jig
47 (Figure 5D) by aligning the specimen stage with the groove in the middle. The recorded parameters
48 should be entered into the 'Nanoeye' software. It is imperative that the height entered is 2-3 mm
49 more than the recorded height to avoid contact of sample to electron gun. In the case of single
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3 aluminum stub (15 mm, 25 mm, or 40 mm in diameter), it can be directly placed on the motorized
4 stage and stage should be fastened using a hex key. Use the 'Camera' button on the Motor Control
5 Panel of software screen to capture the image of the stage as it will later act as a map to capture
6 magnified images of different regions of the specimen. It is important to note that the center of
7 x-y coordinates on the camera navigation should align with the center of the fastened stage. Press
8 'Exchange' switch while keeping the door slightly pushed for few seconds using one hand. After
9 loading a fresh unprepared sample, the user has approximately five minutes to acquire images.

19 **D. Image capturing and processing using 'Nanoeye'**

20 On the Start Page of Nanoeye software screen, select 5KV voltage, SE detector and High vacuum
21 (low vacuum can also be employed depending on sample quality). Then, press 'START' to start
22 scanning of specimen inside vacuum chamber. After pressing start button, check to see that the
23 emission current rises to 110 μ A. The initial page of Image Window shows the fast-scanning mode
24 with minimum resolution. Use X, Y, Z, R, T on Motor Control Panel to select the
25 region/coordinates of sample to be scanned at certain rotation and angle. By default, the Z is equal
26 height of fastened stage, and X, Y, T and R is 0. When selecting X-Y coordinates, double click on
27 camera navigation and later minor changes in the coordinates can be made using X-Y motor
28 operation. Initially, increase the magnification up to 500-1000X to focus rather than focusing at a
29 magnification lower than 500X. Then, decrease the magnification as per requirement and the
30 image to be captured. Select 10-30% spot size on Image Control as per requirement before
31 changing the scan mode. Select the slow photo 2 scan mode (on the extreme bottom right; Image
32 Control) for highest resolution publication quality image. Then select 'Auto' on the
33 brightness/contrast focus area on Image Control. On the bottom bar of Image Window, company
34 label, researcher's name and specimen label can be modified. Monitor the complete scanning on
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3 the screen and just before the scanning is completed, click the 'Camera' icon on bottom right
4 corner of Image Control, and save the image at a designated storage location. To measure the
5 dimensions of the various components in the image, pause the scanning by clicking on pause icon
6 on bottom bar and click M. Tools. Select length to measure two-point/multi-point length and
7 markings (arrow, square, and rectangle) to mark the components of image. Click on the operation
8 button on top right bar of Image control and a dropdown menu will appear. Select 'Stop' to stop
9 scanning. Once 'Stop' is selected on the Nanoeye page, the power switch can be pressed to stop
10 operation of DSEM and Nanoeye window will be closed. A detailed flowchart comparing
11 traditional SEM and DSEM is presented respectively in tables 1 and 2.
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26 **Results and Discussion**

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28 Here we document a detailed procedure of a possible alternative to traditional electron
29 microscopy that removes the major bottlenecks while sustaining image quality. Using SNE- 4500
30 Plus Tabletop Desktop Scanning Electron Microscope, we captured images of fresh leaf samples
31 of different species of Solanaceae (Figure 1B, 1C, 1D, 2A and Figure 3), Cucurbitaceae (Figure
32 1A and 2B), Asteraceae (Figure 2C) and Poaceae (Figure 2D) plant families to study their surface
33 features (for example., trichomes, stomata, and waxes) (Figure 2B). We varied magnifications to
34 estimate the density of trichomes depending on the characteristics of plant families (Figure 2).
35 Additionally, we captured images of insect eggs (Figure 1E; Figure 3A), pollen grains (Figure 1D),
36 and caterpillars using DSEM. Images of fall armyworm (*Spodoptera frugiperda*; Lepidoptera;
37 Noctuidae) and tobacco hornworm (*Manduca sexta*; Lepidoptera; Sphingidae) (Figure 3A) eggs
38 laid by adult moths on tomato (*Solanum lycopersicum*; Solanaceae) leaf surface were collected to
39 assess any damage caused by trichomes present on the leaf surface to eggs.
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6 In DSEM imaging of insect eggs, we did observe some structural distortion (shrinkage)
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8 after placing them under vacuum in DSEM (Figure 3A). However, aldehyde fixing, and sputter
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10 coating would resolve this issue. Previously, Kariyat et al. (2017), had captured images of *M.*
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12 *sexta* caterpillars' peritrophic matrix and frass pellets to study the effects of trichomes of
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14 horsenettle (*Solanum carolinense*: Solanaceae) post-feeding (Kariyat et al., 2017; Figure 4A and
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16 4C). This was done with a traditional Cambridge S360 scanning electron microscope (Huck
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18 institutes of life sciences, Microscopy Core Facility, Pennsylvania State University, USA) and
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20 used typical SEM preparation protocol which includes overnight fixation of the sample at 4°C in
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22 25% glutaraldehyde solution, dehydration rinses through series of ethanol solutions, critical point
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24 drying, and sputter coating of critically dried sample (Figure 4A and 4C; Table 1). However,
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26 without using the abovementioned method, we captured images of frass pellets of cabbage loopers
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28 (*Trichoplusia ni*; Lepidoptera: Noctuidae) to detect the presence of undigested trichomes when the
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30 caterpillars were fed on cucumber (*Cucumis sativa*; Cucurbitaceae) and bottle gourd (*Lagenaria*
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32 *sicerraria*; Cucurbitaceae) leaves. Interestingly, we obtained similar results of the trichomes being
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34 embedded in these frass pellets (Figure 1F; Figure 4B, 4D) to the images earlier captured by
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36 Kariyat et al. (2017). Additionally, plant waxes, one of first line of defenses encountered by
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38 herbivores, were also observed very clearly in our SEM images from *Solanum glaucescens* (Figure
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40 1B). Additionally, the resolution of DSEM is 5nm, comparable to traditional SEMs with nanoscale
41
42 resolution. This strengthens our point that DSEM is equally capable of capturing minute details
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44 from the specimens with no sample prep which was earlier thought to be only captured by
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46 expensive high-tech SEMs which use extensive sample preparation methodology.
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3 While sample preparation and image acquisition are much easier and can be accomplished
4 quickly, there are some concerns. We found that it is difficult to estimate the gland cell number
5 and the exact shape of the gland of glandular trichomes, and eggs of insects due to the damage
6 sustained after placing the sample under vacuum in DSEM (Figure 3). Alternatively, we found that
7 shape of non-glandular trichomes of tomato were not distorted under vacuum. Sputter coating and
8 would have possibly produced better images of glandular trichomes. Using a more efficient
9 method for capturing scanning electron microscope images by placing the sample on the stage can
10 produce similar results as produced by using more complicated and traditional SEM. This is
11 especially true for plant-insect interactions studies where routine imaging of plant and insect parts
12 could be fast tracked using DSEM. Overall, we demonstrate that using DSEM, which is fast, easy
13 to operate, and inexpensive, can produce quality images similar to other SEM, with significantly
14 lower costs of purchase, maintenance and methodology.
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40 Conflict of Interest

41 The authors declare that there are no personal, professional, or financial conflicts among them
42 that have or can affect the work of described in the manuscript.
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TRADITIONAL SCANNING ELECTRON MICROSCOPY

- Fixation of sample: 2.5% glutaraldehyde in phosphate buffer
- Post fixation: three changes in phosphate buffer which takes 5 min
Placing sample in 1% osmium tetroxide
- Dehydration – serial dehydration each of 25%, 50%, 70 %, 80%. 90% and 100%, each at 30 min (varies with sample type)
- Critical point drying: slide is transferred to critical point apparatus
liquid carbon dioxide is passed through the sample and temperature is raised to 34C, raised to 1280 Psi
- Sputter coating: heavy metal coating with gold and palladium/ Vacuum evaporation coating
- Transferring the specimen to the specimen stub with the help of liquid adhesives such as cyanoacrylate or conductive tapes such as carbon and sticky tabs
- The machine is under vacuum; Clicking vent switch in software to de-vacuum Scanning electron microscope (SEM)
- Placing the sample stub in the specimen holder (9 samples can be processed at a time)
- Adjust working distance
- Close the SEM door and turn on the vacuum
- Turn on the electron beam
- Image is focused and magnified
- Saturation of electron beam must be self-adjusted
- To obtain high resolution image, SEM involve technical expertise and playing with other different parameters such as wobble and stagnation.

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5 **Tables 1: A flowchart representing the basic steps involved in image**
6 **acquisition on traditional SEM**
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DESKTOP SCANNING ELECTRON MICROSCOPY

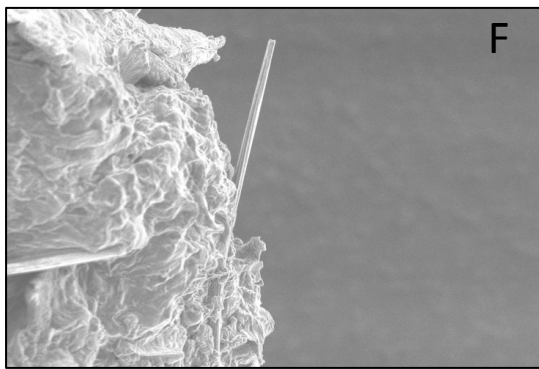
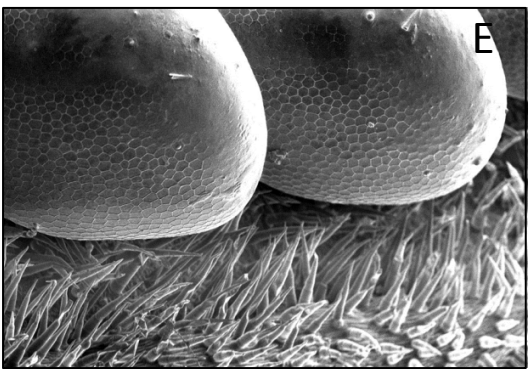
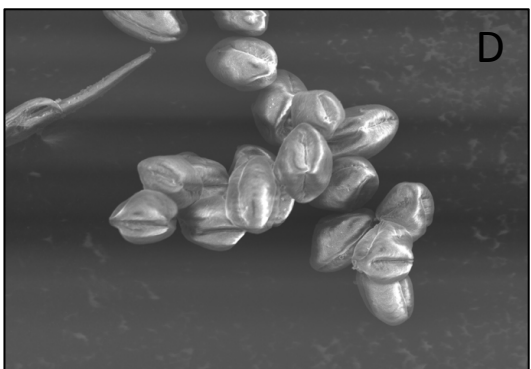
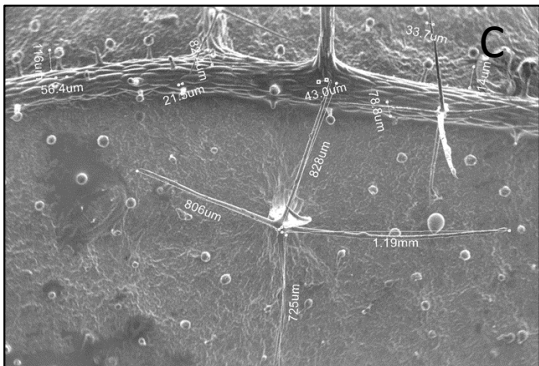
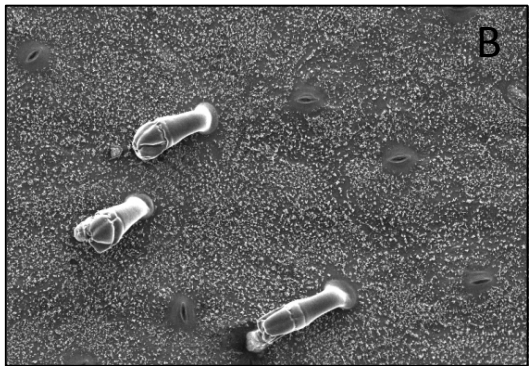
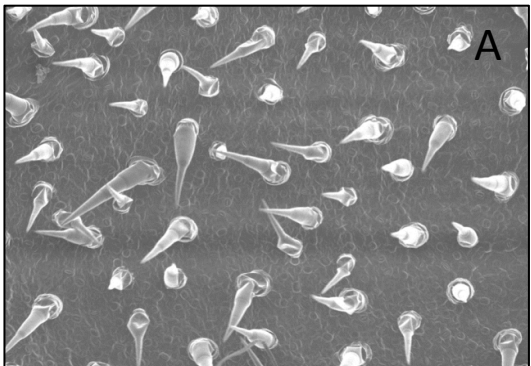
- 1. Pump should be operating with a pressure of 90-120 psi
- 2. Before de-vacuuming the DSEM, build a complete vacuum which is indicated by vacuum LED filled with green light
- 3. After de-vacuuming, slide the chamber door to take out the stage until you hear a 'click' sound
- 4. Open 'Nanoeye' software and the window displayed can be divided into three separate sections: Motor Control Panel- controls the motor and navigates samples; Image Window- displays image of the specimen; Start Page- customize basic settings for imaging before imaging before starting scanning of sample
- 5. Measure the height and width of sample stage and enter them in Motor Control of 'Nanoeye' software before putting the sample on the stage and inserting it into vacuum chamber
- 6. Prepare the sample stage by fixing fresh biological/non-biological sample on double-sided carbon tape
- 7. After placing the sample stage in the vacuum chamber and building the vacuum completely, press 'Start' on Start Page and when emission current reaches 110 μ A, the entire window can be divided into three separate sections: Motor Control; Image Window; Image Control- adjusts the focus, spot size, brightness and contrast of the specimen image, and images at different scanning modes can be captured,
- 8. For measuring specific structures in the sample, scanning is paused, and measurements can be recorded by selecting different shapes from 'M. tools' menu. After measurement, images can be captured by clicking camera icon on Image Control and saved at desired location.



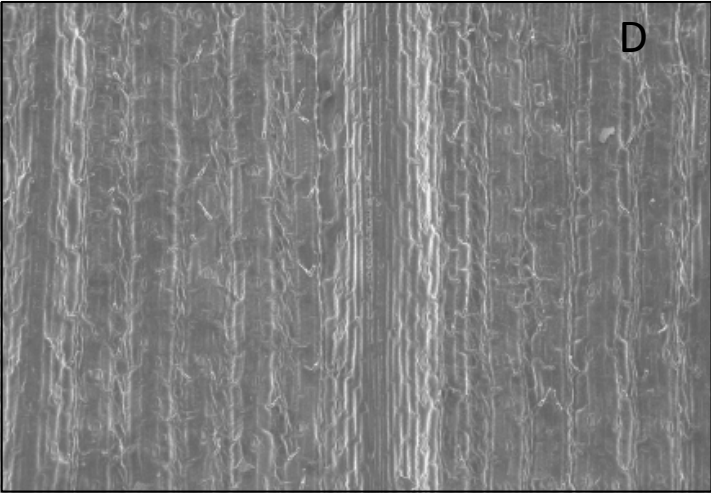
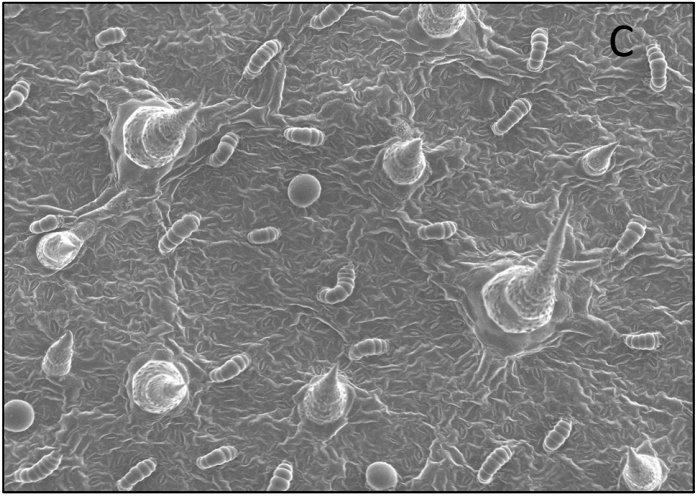
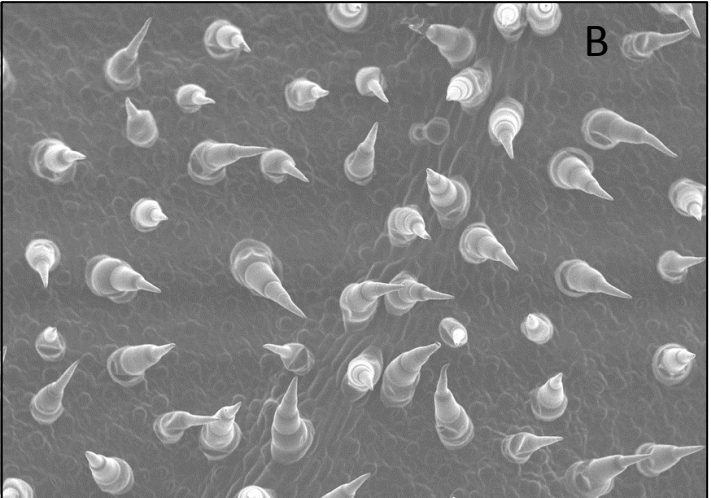
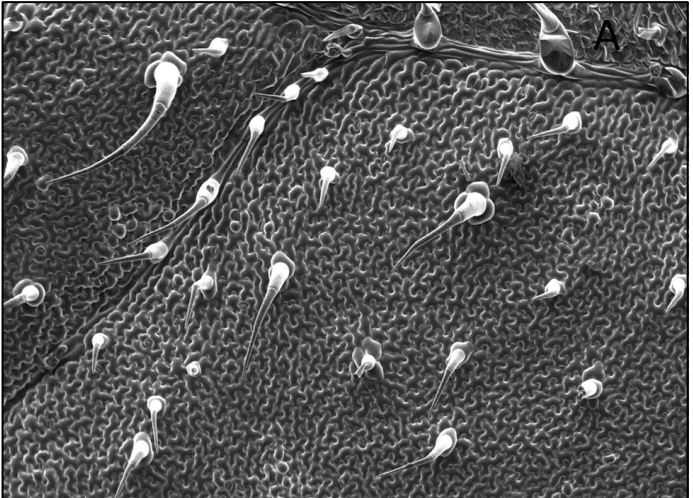
TAKES FIFTEEN MINUTES

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5 Table 2: A flowchart representing the basic steps involved in image
6 acquisition on SNE- 4500 Plus Tabletop SEM with representative
7 images of SNE- 4500 parts.
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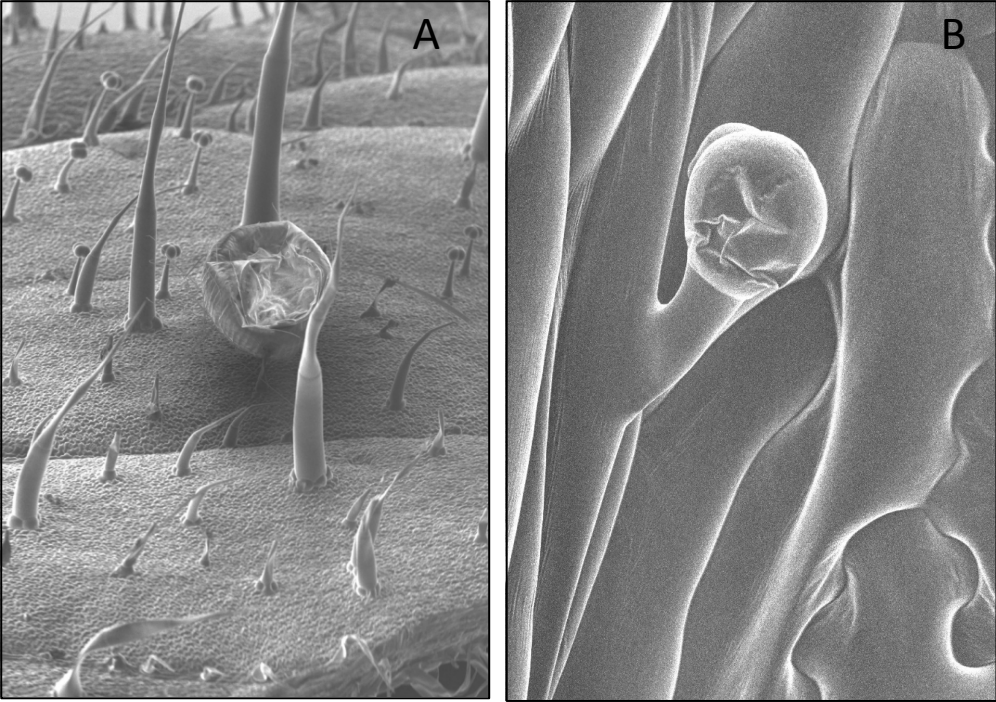
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Figure 2. Desktop Scanning electron microscopic images of various kinds of trichomes present in different plant families including (A) Solanaceae (tomato; *Solanum lycopersicum*) at 140X, (B) Cucurbitaceae (bottle gourd; *Lagenaria siceraria*) at 200X, (C) Asteraceae (sunflower; *Helianthus annuus*), and (D) Poaceae (sorghum; *Sorghum bicolor*)

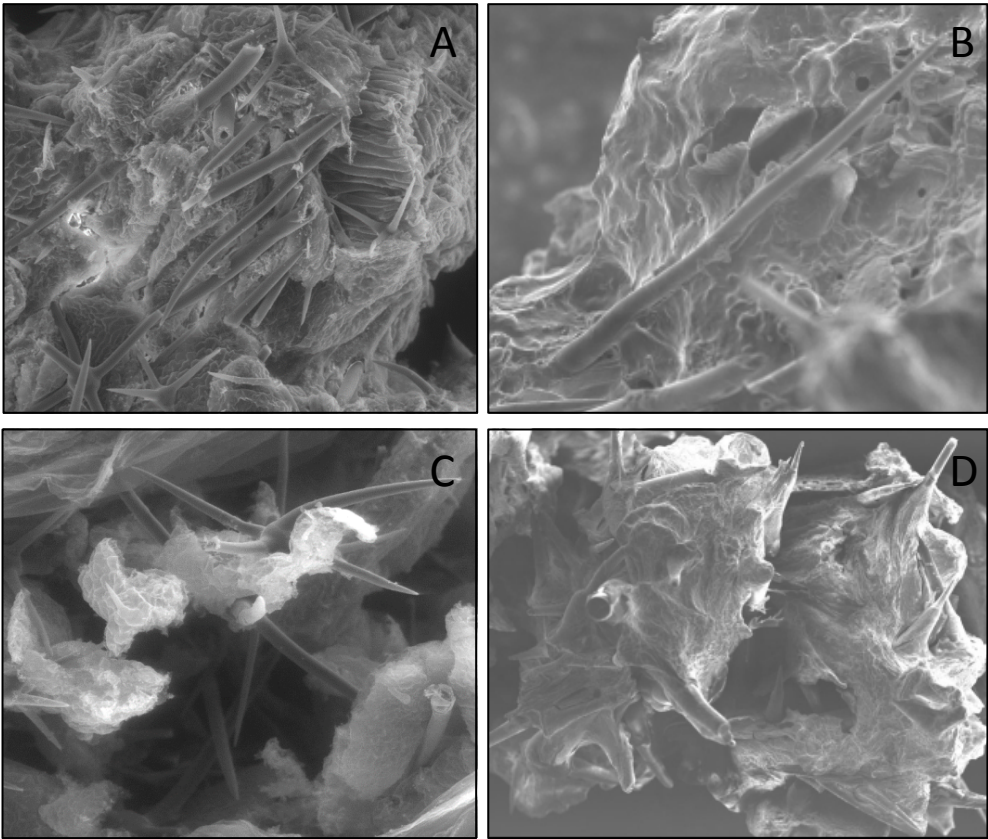
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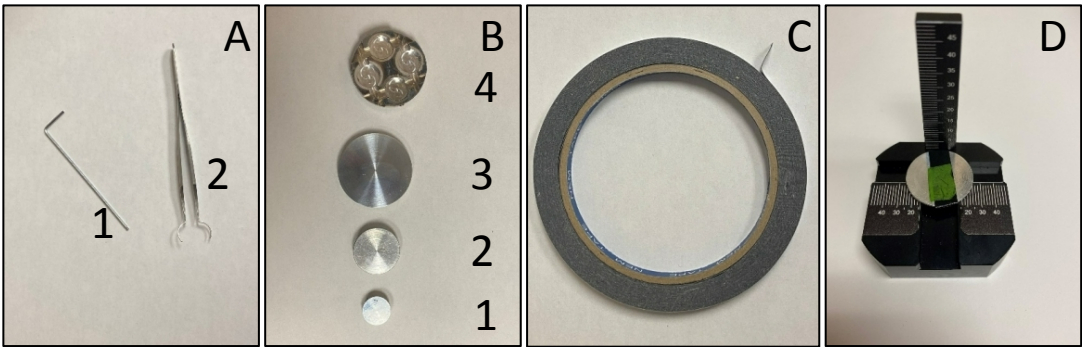
Figure 3. Desktop scanning electron microscopy showing shrunken (A) egg of fall armyworm (*Spodoptera frugiperda*) on tomato (*Solanum lycopersicum*; Solanaceae) leaf surface at 75X, and (B) glandular trichome of African eggplant (*Solanum macrocarpon*; Solanaceae) at 1000X, indicating that the although the surface features of a biological sample are visible, but not fixing sample and leaving out sputter coating can lead to deviation of image from its original structure.

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4 Figure 4. Scanning electron microscopy (SEM) of undigested trichomes embedded in frass
5 pellets of (A and C) tobacco hornworm (*Manduca sexta*: Sphingidae) caterpillar fed on
6 trichome rich plant material of horsenettle (*Solanum carolinense*; Solanaceae). Scanning of
7 sample was carried out at 300X and 400X using traditional Cambridge S360 scanning
8 electron microscope following conventional sample fixation with gluteraldehyde, tissue
9 dehydration with ethanol washes, critical point drying of sample and sputter coating, and (B
10 and D) cabbage looper (*Trichoplusia ni*: Noctuidae) after caterpillar fed on cucumber
11 (*Cucumis sativa*; Cucurbitaceae) and bottle gourd (*Lagenaria Sicerraria*; Cucurbitaceae)
12 plant material and scanning of sample done at 450X and 150X using no sample preparation
13 desktop scanning electron microscope.
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7 **Figure 5. Frequently used tools for sample preparation and handling while**
8 **operating Desktop Scanning Electron Microscope: (A) 1. Hex key to loosen or**
9 **tighten the screws of stage while taking out or putting the sample stage**
10 **(aluminum stub with sample mounted on it) in vacuum chamber 2. SEM**
11 **mount forceps to handle the aluminum stubs without damaging the sample,**
12 **(B) Aluminum stubs of diameter 1. 15 mm, 2. 25 mm, 3. 40 mm and 4. 40**
13 **mm stub to handle multiple (1-4) 15mm stubs at one time, (C) Double-sided**
14 **carbon tape used to fix sample on the aluminum stubs, and (D) Machine Jig**
15 **to measure the diameter and height of sample stage.**
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