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OPTICAL SECTIONING AND 3D RECONSTRUCTIONS AS AN ALTERNATIVE TO SCANNING ELECTRON MICROSCOPY FOR ANALYSIS OF CELL SHAPE¹

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- *Premise of the study:* Visualizing flower epidermal cells is often desirable for investigating the interaction between flowers and their pollinators, in addition to the broader range of ecological interactions in which flowers are involved. We developed a protocol for visualizing petal epidermal cells without the limitations of the commonly used method of scanning electron microscopy (SEM).
- *Methods:* Flower material was collected and fixed in glutaraldehyde, followed by dehydration in an ethanol series. Flowers were dissected to collect petals, and subjected to a Histo-Clear series to remove the cuticle. Material was then stained with aniline blue, mounted on microscope slides, and imaged using a compound fluorescence microscope to obtain optical sections that were reconstructed into a 3D image.
- *Results:* This optical sectioning method yielded high-quality images of the petal epidermal cells with virtually no damage to cells. Flowers were processed in larger batches than are possible using common SEM methods. Also, flower size was not a limiting factor as often observed in SEM studies. Flowers up to 5 cm in length were processed and mounted for visualization.
- *Conclusions:* This method requires no special equipment for sample preparation prior to imaging and should be seen as an alternative method to SEM.

Key words: confocal microscopy; fluorescence; morphology; optical sectioning; petal cells.

Visualization of flower epidermal cells has been employed in many different types of studies ranging from development to ecological studies. The functions of these cells are known as major contributors for flower development, pollinator success, flower wettability, micromorphological markers for petal identity, and cellular determinants of flower color (Kay et al., 1981; Glover and Martin, 1998; Ojeda et al., 2009; Whitney et al., 2011; Landis et al., 2012; Ojeda et al., 2012; Puzey et al., 2012). Most of these studies used scanning electron microscopy (SEM), with a few using only light microscopy, or light microscopy as a standard to compare with SEM images. None of these methods are without limitations, but they are often preferred over methods for imaging cellular structure that involve conventional histological techniques such as embedding material in wax followed by microtome sectioning (Feder and O'Brien, 1968).

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Despite its benefits for many applications, SEM has some limitations for analysis of floral epidermal cells. Sample preparation requires a dehydration step, often with critical point drying or freeze-drying. Critical point drying requires a specialized apparatus, has limited throughput (Araujo et al., 2003), and can cause shrinkage of biological material and violent bubbling (Boyde and Wood, 1969; Sargent, 1983). Although freeze-drying can keep the waxy cuticle intact, the underlying cells may not be distinguishable (Sargent, 1983). Additional damage can also be caused by vacuum pressure in the SEM or beam damage while acquiring the image (Eveling, 1984). A variety of alternative techniques, as well as many comparisons of techniques, have been reported, but inconsistencies arise due to individual aptitude or equipment (Pathan et al., 2008). Several studies have compared different SEM sample preparation methods to determine which methods produce the fewest artifacts (Parsons et al., 1974; Sargent, 1983; Ensikat et al., 2010).

Our objective was to develop a protocol for visualizing flower cells that allows for higher throughput than SEM, does not require special equipment for sample preparation prior to imaging, bypasses some of the limitations often encountered with SEM (most notably cell damage and constraints on the size of tissue to be imaged), and does not have the same size limitations as SEM. Most studies utilizing SEM use samples that range in size from 3–5 mm (Eveling and McCall, 1983; Sargent, 1983; Eveling, 1984). This arises as a limitation on the size of the stub onto which the specimen is mounted for visualization in the SEM. Flowers that exceed this size often need to

be dissected and individual pieces processed before mounting on stubs (Pathan et al., 2008). This requirement can further damage tissue, as well as cause orientation problems if viewing and analysis of the whole flower are desired.

The approach we propose here differs from other previous methods for visualizing floral cells because many of the preparation procedures that have been shown to have the largest impact on cell structure are avoided with this method; material is fixed before staining and imaging, thus samples do not need to be visualized immediately after collection. Larger samples (up to 5 cm) can also be imaged, and no special equipment is necessary to prepare the samples before imaging. Imaging of prepared material can be conducted on any microscope that can produce optical sections, including compound fluorescence wide-field microscopes equipped with structured illumination (ApoTome; Zeiss, Jena, Germany), confocal laser-scanning microscopes, spinning disc confocal microscopes, and light-sheet fluorescence microscopes.

METHODS

Taxon sampling—Petal cells were imaged and measured for four flower developmental stages for three species of *Petunia* Juss. (Solanaceae: *P. axillaris* (Lam.) Britton, Sterns & Poggenb. [PI 667515], *P. exserta* Stehmann [OPGC 943], and *P. integrifolia* (Hook.) Schinz & Thell. [PI 667517]), 11 taxa of *Gilia* Ruiz & Pav. (Polemoniaceae: *G. angelensis* V. A. Grant [RSABG 21065], *G. brecciarum* M. E. Jones subsp. *brecciarum* [W6 30785], *G. cana* (M. E. Jones) A. Heller subsp. *speciformis* A. D. Grant & V. E. Grant [RSABG 17663], *G. capitata* Sims subsp. *abrotanifolia* (Nutt. ex Greene) V. E. Grant [RSABG 22495], *G. clokeyi* H. Mason [W6 30789], *G. inconspicua* (Sm.) Sweet [W6 30132], *G. leptantha* Parish [RSABG 21365], *G. nevinii* A. Gray [RSABG 18895], *G. sinuata* Douglas ex Benth. [RSABG 16717], *G. tenuiflora* Benth. [RSABG 17191], and *G. tricolor* Benth. subsp. *diffusa* (Congdon) H. Mason & A. D. Grant [RSABG 17613]), and four taxa of *Salpiglossa* (V. E. Grant) L. A. Johnson (Polemoniaceae: *S. australis* (H. Mason & A. D. Grant) L. A. Johnson [Leigh Johnson, BYU], *S. caruifolia* (Abrams) L. A. Johnson [RSABG 19148], *S. splendens* (Douglas ex H. Mason & A. D. Grant) L. A. Johnson subsp. *grantii* (Brand) L. A. Johnson [RSABG 21757], and *S. splendens* subsp. *splendens* [RSABG 22676]). All material was grown in greenhouses at the University of Florida from seeds obtained through Rancho Santa Ana Botanic Garden (RSABG) and the Ornamental Plant Germplasm Center (OPGC, PI, and W6).

Tissue fixation and preparation—A protocol for fixing and staining flower material was modified from previously published protocols for visualizing cells using different platforms (SEM: Landis et al., 2012; confocal: Bougourd et al., 2000) (Appendix 1). Fresh whole flowers were collected and fixed in a glutaraldehyde and phosphate buffer solution consisting of 2% electron microscopy-grade glutaraldehyde (Electron Microscopy Sciences, Hatfield, Pennsylvania, USA) and 240 mM phosphate buffer (31.6 mL of 1 M sodium phosphate monobasic and 68.4 mL of 1 M sodium phosphate dibasic brought up to 1 L with a pH of 7.4). Batches of smaller flowers were fixed in 50 mL of fixative solution in conical polypropylene screw-capped centrifuge tubes. Flowers were left in solution at 4°C for a minimum of 1 wk and a maximum of 4 wk, with larger flowers needing more time than smaller flowers. Fixation was allowed to proceed until flowers had lost most of their pigment. The fixative was removed, and the flower material was dehydrated through an ethanol/water (v/v) series (50%, 70%, 85%, 95%) for a minimum of 1 h at each stage and stored in 95% ethanol. The 50% and 70% stages were carried out at -20°C because colder ethanol has been shown to work best for dehydrating samples (Feder and O'Brien, 1968). The remainder of the ethanol series was conducted at 4°C. Flowers were transferred to 100% ethanol before longitudinal sectioning and removal of sepals, stamens, and carpels. Petals were transferred to glass scintillation vials. From this stage onward, all steps were conducted at room temperature. For cuticle removal, dissected flower material was moved through a Histo-Clear (National Diagnostics, Atlanta, Georgia, USA) series (v/v, Histo-Clear/ethanol) (25%, 50%, 75%, 100%) for 1 h at each stage. To remove Histo-Clear, the flower material was processed through an ethanol/Histo-Clear (v/v) series (25%, 50%, 75%, 100%) and finally rehydrated in a water/ethanol (v/v) series (25%, 50%, 75%, 100%).

Flower material was vacuum-infiltrated twice for 10 min in 1% (w/v) aniline blue in phosphate-buffered saline (PBS) solution (8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, and 0.24 g KH₂PO₄ brought up to 1 L with a pH of 7.4). Material was left in staining solution for at least one week, but usually up to a month before mounting on slides.

Mounting—Stained petals were mounted on microscope slides using fluorescent mounting media (Kirkegaard & Perry Laboratories, Gaithersburg, Maryland, USA). Cover slips were sealed with commercial-grade clear fingernail polish. Final prepared slides were stored at 4°C and wrapped in aluminum foil to reduce light exposure.

Optical sectioning—Fluorescent images from prepared slides were collected with a Zeiss AxioCam high-resolution microscope camera mounted on a Zeiss AxioPlan 2 Imaging microscope. Green fluorescence was obtained using Zeiss filter set 10 (excitation wavelengths, 450–490 nm; dichroic, 510 nm LP; emission wavelengths, 515–565 nm), a 40× magnification lens, and ApoTome with optical sectioning. Z-stacks were acquired using AxioVision software (Zeiss) with default optimal slice distance of 0.675 μm (about 10–15 sections per stack). Stacks were then imported into Fiji (<http://fiji.sc/Fiji>; Schindelin et al., 2012) for further analyses of cell size, circularity, and number. For clear delimitation of cells during analysis, composite images were created from the imported Z-stacks in Fiji using maximum intensity, while 3D images were created using the AxioVision software.

SEM comparison—SEM samples were prepared following the method described in Landis et al. (2012). Fresh tissues were collected and fixed using the same method as above. Samples were dehydrated, processed in a Tousimis critical point dryer (Rockville, Maryland, USA), and sputter-coated using a Denton Desk V sputter coater (Denton Vacuum, Moorestown, New Jersey, USA). Samples were then imaged using a Hitachi S-4000 FE-SEM (Minato-Ku, Tokyo, Japan).

RESULTS

In flowers of *Salpiglossa*, cell walls of all of the cells were visible using this optical sectioning method followed by 3D reconstruction, in contrast to our previous analyses using SEM. The inability to distinguish certain cell types with SEM has been reported previously (Pathan et al., 2008; Ojeda et al., 2009). Our own SEM analysis of flowers of the same *Salpiglossa* species showed clear differentiation of conical cells on the petal lobes, but cells toward the base of the petal tube were indistinguishable with SEM but clearly visible using this optical sectioning protocol (Fig. 1).

Preparation of samples is more convenient and cost effective with our optical sectioning–3D reconstruction method than for most methods for SEM, especially because no special equipment is necessary for sample preparation prior to imaging. This aspect also reduces the cost of sample preparation, especially if researchers are charged per run for use of a critical point dryer or sputter coater. Samples can also be mounted on regular microscope slides with a cover slip instead of on SEM stubs and carbon paper, allowing for analysis of larger floral tissues. SEM can typically accommodate samples ranging from 3–5 mm (Eveling and McCall, 1983; Sargent, 1983; Eveling, 1984), but with the described method, flowers up to 5 cm could be mounted on a single slide. Flowers longer than 5 cm were sectioned once, with orientation of the pieces being straightforward. Labeling microscope slides is also much easier than SEM stubs. With the initial fixative step, samples can be collected and then stored until imaging. Slides of stained petals can stay in stain for months with no adverse effects. Once samples are mounted on slides and sealed properly, they can be stored at 4°C for at least several months before image processing. The largest issue with slide storage is ensuring complete sealing of the cover slips on slides. Sealing the slides is critical if samples need to

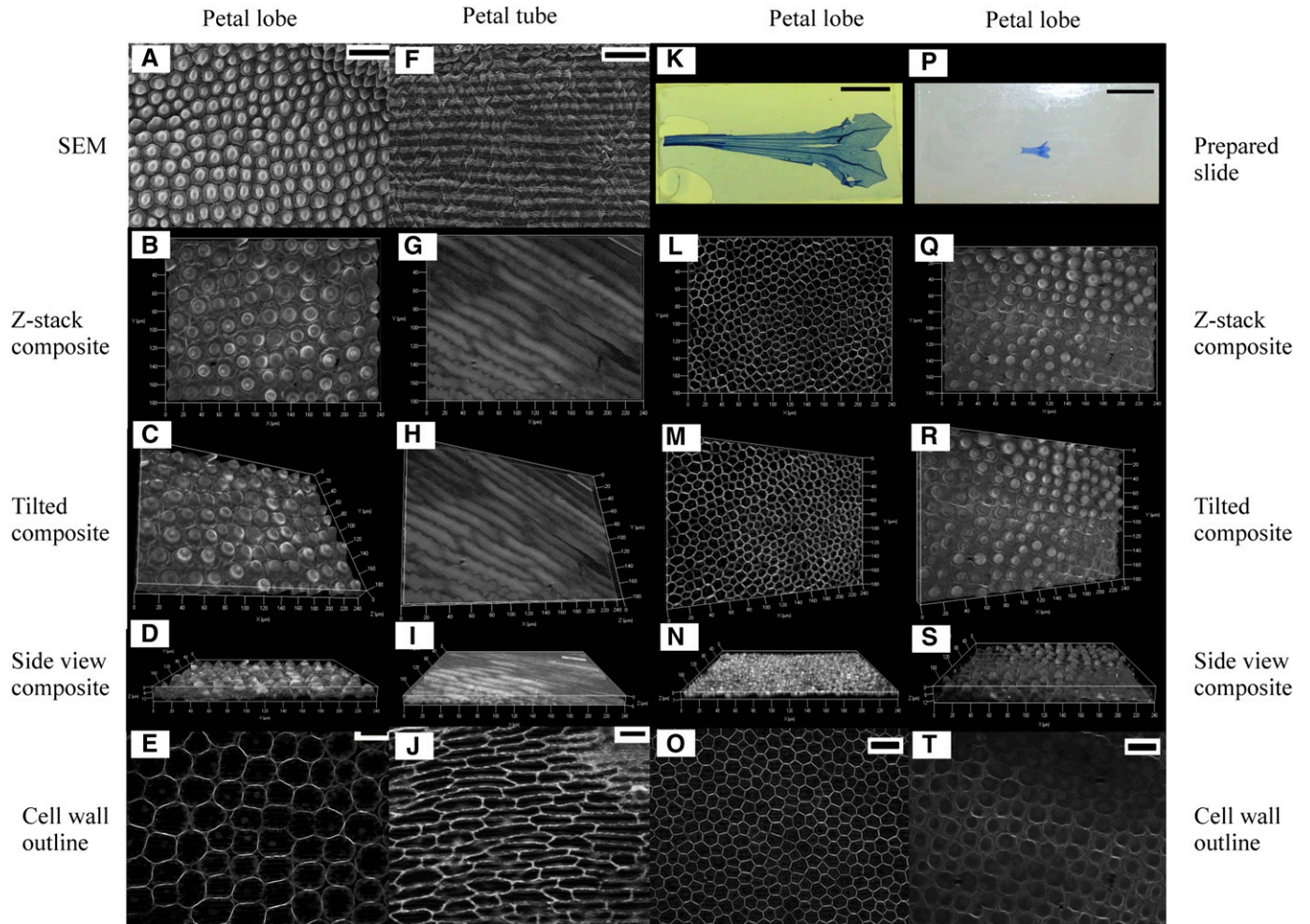


Fig. 1. Comparison of SEM and optical sectioning images of petal lobe (A–E) and tube (F–J) cells obtained from *Saltugilia* taxa, as well as additional comparison of petal lobes in *Petunia* (K–O) and *Gilia* (P–T) to show the utility among different genera. (A) and (F) are from samples fixed using glutaraldehyde and critical point drying for SEM. The rest were obtained following the method outlined and imaged on a compound fluorescence microscope. (A) SEM of *S. splendens* subsp. *splendens*, (B) Z-stack composite of *S. caruifolia*, (C) tilted composite of *S. caruifolia*, (D) side view of composite *S. caruifolia*, (E) cell outlines of *S. splendens* subsp. *splendens*, (F) SEM of *S. splendens* subsp. *splendens*, (G) Z-stack composite of *S. caruifolia*, (H) tilted composite of *S. caruifolia*, (I) side view of composite *S. caruifolia*, (J) cell outlines of *S. splendens* subsp. *splendens*, (K) prepared slide of *Petunia axillaris*, (L) Z-stack composite of *P. axillaris*, (M) tilted composite of *P. axillaris*, (N) side view of composite *P. axillaris*, (O) cell outlines of *P. axillaris*, (P) prepared slide of *Gilia sinuata*, (Q) Z-stack composite of *G. sinuata*, (R) tilted composite of *G. sinuata*, (S) side view of composite *G. sinuata*, (T) cell outlines of *G. sinuata*. Scale bars: A and F = 87.5 μm , E and J = 20 μm , K and P = 1 cm; rulers illustrate scale for 3D stacks.

be imaged multiple times. Initial analyses indicate that all of the fixative and preparation steps are necessary. When the Histo-Clear steps were not followed, cell walls were not evenly and consistently stained. Following all of the steps yields clearer images than using DAPI (4',6-diamidino-2-phenylindole), or the autofluorescence caused by glutaraldehyde (results not shown). Slides were also imaged on a Leica TCS SP5 confocal laser-scanning microscope (CLSM; Leica Microsystems, Buffalo Grove, Illinois, USA) with a 63 \times oil objective and excitation by the 543 line of the HeNe laser at 100% power yielding the same quality images as shown, which demonstrates the flexibility and utility of this method for imaging cells (Appendix S1).

The literature is rich with different methods for visualizing cell structure. Parsons et al. (1974) compared 12 different sample preparation methods for visualizing cells and found that with no time or equipment constraints, the best method was the

use of untreated material. However, this approach requires that tissue be examined immediately after collection, which in some cases may not be feasible. Confocal microscopy is valuable for a variety of botanical applications (Hepler and Gunning, 1998), including analyses of stamen formation in maize (Gao et al., 2013) and plant tissue infected with fungal hyphae (Doehlemann et al., 2009). Additionally, using ultraviolet excitation can increase the capacity of cellular visualization (Fricker and White, 1992). A more recent study found that tissue fixation and dehydration with methanol was a better method than other fixation and dehydration protocols; however, this method still requires the use of SEM preparation equipment (Talbot and White, 2013). A similar method using aniline blue was used for visualizing *Arabidopsis* embryos, but this method required clearing samples with chloral hydrate (Bougourd et al., 2000). Other recent methods have also used periodic acid–Schiff method (Raczyńska-Szajgin and Nakielski, 2014) or Bis(trimethylsilyl)amine

(hexamethyldisilazane, HMDS) solvent instead of critical point drying (Araujo et al., 2003).

This optical sectioning–3D reconstruction method can have potential drawbacks. Photobleaching during image capture occurred rarely if the intensity of the laser was too strong or if samples were too thick, as mentioned by Hepler and Gunning (1998). During mounting, if the cover slips are pressed down on the material the cells will collapse. This problem can be easily rectified with a variety of methods, including using specialized slides that have slightly raised edges.

CONCLUSIONS

This optical sectioning–3D reconstruction method of cell visualization yields high-quality images of cells with a cost-efficient approach with no special equipment needed prior to imaging. Even though only petal cells were visualized with this method, we believe this method is applicable to a wide range of tissues (such as leaves, sepals, and stamens), as well as a wider range of angiosperm diversity than presented here. This preparation method can also be coupled with different types of microscopes to yield high-quality images, as demonstrated with the use of multiple microscopes presented here. There are no constraints on timing, because samples can be held at many points for as long as necessary with no adverse effects, including the initial fixative stage and the final staining stage. With this method, all cell types of the petal epidermis were easily distinguishable by their cell walls. In addition, 3D plots and composite images can be generated with a single capture of images with no adjustment of the material in any way. Both of these characteristics are often desired for determining overall cell shape in investigations of possible ecological interactions.

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APPENDIX 1. Materials list and protocol for optical sectioning–3D reconstruction method.

Materials needed:

Aniline blue (catalog no. 415049-25g; Sigma-Aldrich, St. Louis, Missouri, USA)
Ethanol
Fingernail polish
Fluorescent mounting media (catalog no. 71-00-16; Fisher Scientific, Waltham, Massachusetts, USA)
Glutaraldehyde (catalog no. 16320; Electron Microscopy Sciences, Hatfield, Pennsylvania, USA)
Histo-Clear (catalog no. HS-200; National Diagnostics, Atlanta, Georgia, USA)
Potassium chloride
Potassium phosphate dibasic
Sodium chloride
Sodium phosphate dibasic dodecahydrate
Sodium phosphate monobasic monohydrate

Solution recipes:

1 M phosphate buffer:

- Add 68.4 mL of 1 M Na₂HPO₄ dodecahydrate + 31.6 mL of 1 M NaH₂PO₄ monohydrate to 900 mL dH₂O (pH 7.2)
- Autoclave to sterilize

1× PBS buffer:

- 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄ anhydrous, 0.24 g KH₂PO₄ dibasic
- Dissolve reagents in 800 mL H₂O. Adjust pH to 7.4 with HCl, add H₂O to 1 L final volume. Autoclave.

2% glutaraldehyde in 1 M phosphate buffer:

- Glutaraldehyde comes in 50% solution in 10 mL
- 10 mL glutaraldehyde + 240 mL 1 M phosphate buffer = 250 mL total

Protocol:

- Fix for ≥ 2 h in solution, preferably 1 h on the bench, overnight at 4°C.
- Usually use 50 mL of fixative to fix two to three large, full, open flowers in 50-mL conical polypropylene screw-capped centrifuge tubes.
- Use fresh glutaraldehyde fixative.
- Make sure all tissue is completely submerged; may need to put Kimwipes (Kimberly Clark Professional, Roswell, Georgia, USA) in the top of polypropylene tubes to push all material down far enough to be completely submerged.

1. After overnight fixation, drain off fixative and put tissue through ethanol series as follows:

50% (v/v) ethanol for 1 h 30 min at –20°C
70% (v/v) ethanol for 1 h 30 min at –20°C
85% (v/v) ethanol for 1 h at 4°C
95% (v/v) ethanol for 1 h at 4°C
95% (v/v) ethanol overnight at 4°C
100% ethanol for 1 h at 4°C
100% ethanol overnight at 4°C
100% ethanol for 1–2 h at room temperature

At this stage flowers are easiest to cut longitudinally for ease of mounting onto slides. Transfer targeted material glass scintillation vials for further processing.

2. When trying to remove the waxy cuticle, move through Histo-Clear (National Diagnostics) series:

25% (v/v) Histo-Clear/ethanol for 1 h
50% (v/v) Histo-Clear/ethanol for 1 h
75% (v/v) Histo-Clear/ethanol for 1 h
100% (v/v) Histo-Clear for 1 h, two times

3. Bring back into ethanol:

75% (v/v) Histo-Clear/ethanol for 1 h
50% (v/v) Histo-Clear/ethanol for 1 h
25% (v/v) Histo-Clear/ethanol for 1 h
100% ethanol for 1 h, two times

4. Rehydrate samples:

75% (v/v) ethanol for 1 h
50% (v/v) ethanol for 1 h
25% (v/v) ethanol for 1 h
Water for 1 h, two times

5. If vacuum infiltrating, pull vacuum for 10 min in 1 : 10 dilution of aniline blue and 1× PBS buffer. Replace with new mixture, and repeat 10 min.

6. Let tissue sit in stain overnight (longer yields no adverse effects) in 1 : 10 dilution of aniline blue and 1× PBS buffer.

7. Mount flower material onto microscope slides using three or four drops of fluorescent mounting media. Place cover slip on top, without pressing down. Seal slide using fingernail polish.

8. Store at 4°C wrapped in aluminum foil. Image samples within two weeks.
