**SEM sample preparation protocol – for plant tissue**

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1. **Fixation**

Samples have to be fixed to maintain the cellular structures. Fixation stops all biological activities in the cell.

If you are not going to process the samples immediately, put fresh samples in 1% glutaraldehyde, 4℃ for long term storage. Unlike animal tissue, plants usually have air space within tissue, and the cuticle or wax on surface will block fixatives to filtrate in to tissue or cells, so the samples in the fixatives have to be vacuumed in order to evacuate the air and to facilitate the filtration. Put samples in fixative and slightly loose the lid in vacuum chamber, turn on the pump until you see the small bubbles from the tissue. Turn off the pump, keep the chamber in vacuum condition and let samples stand for 10 to 15 mins, then release the pressure. Repeat several times until samples sink to the bottom. Parafilm the sample vials and store samples at 4℃ .

If you can process the samples right away, put the fresh tissue in Karnovsky’s fixative (recommended by USIF. Alternatively, you can use 2.5 % glutaraldehyde). If your samples are stored in 1% glutaraldehyde, simply change the solution to Karnovsky’s fixative. The volume of fixative should be at least 7 times of the sample tissue. The fixation time depends on the size of the tissue. The sample should not thicker than 1mm at least in one dimension. Usually, samples are in fixative for 2 to 4 hours in room temperature with gently shaking speed on shaker. 4℃, overnight is also acceptable.

1. **Dehydration**

All steps below are in room temperature and sample should be put on shaker.

Use phosphate buffer to rinse the fixed samples before ethanol dehydration. Start ethanol dehydration series: 30%, 50%, 70%, 80%, 90%, 95%, 100%. The dehydration time for each step also depends on tissue size. Usually 10 to 30 mins for each step. Sample can stay in 70%, 4℃ for few days, so here is a good stop point for sample preparation. Put samples in 100% ethanol overnight (can’t stay longer) before critical point dry.

**Summary of protocol:**

Karnovsky’s fixative 2-4 hr, RT

0.1M Phosphate buffer 10 min x 3, RT

30% Ethanol 10-30 min, RT

50% Ethanol 10-30 min, RT

70% Ethanol 10-30 min, RT (can stop here, O/N in 4℃)

80% Ethanol 10-30 min, RT

90% Ethanol 10-30 min, RT

95% Ethanol 10-30 min, RT

100% Ethanol 10-30 min, RT

100% Ethanol Overnight, RT

**Chemical recipes:**

For 100mL, 1% Glutaradehyde:

50% Glutaradehyde EM grade 2 mL

0.2 M Sorensens Phosphate Buffer, pH 7.2 48 mL

Distilled Water 50 mL

\*in dark bottle, and parafilmed, 4℃

For 100 mL, half-strength Karnovsk’s fixative:

16% Paraformaldehyde Solution 13 mL

50% Glutaraldehyde EM Grade 5 mL

0.2M Sorensens Phosphate Buffer, pH 7.2 50 mL

Distilled Water 32mL

\*in dark bottle, and parafilmed, 4℃

For 0.1 M Phosphate buffer:

Dilute 0.2 M Sorensens Phosphate Buffer to 0.1M by distilled water. (store in 4℃)

1. **Critical point drying (CPD)**

Water has high surface tension, and when it leaves tissue, it will break the tissue. CPD can prevent this happening and keep the tissue in good shape. Using liquid CO2 as transitional fluid and increase temperature and pressure to critical point, the liquid can change to gas without destroying the tissue. Water is not compatible with liquid CO2, so here we use 100% ethanol as intermediate fluid. USIF has a critical point dryer, you have to contact USIF before use it.

1. **Coating**

SEM detects secondary electrons from the surface of samples to visualize the surface. Biological samples is non-conductive, so it requires a thin layer of metal coating. After CPD, mount the samples on the stubs and then do the sputter coating.