**BEFORE YOU START**

**It is expected that you are starting this protocol with tissues fixed in a 2.5% glutaraldehyde / 2.5% formaldehyde / 0.1M Sodium Cacodylate buffer pH 7.2 solution. I usually collected samples in these shell vials from electron microscopy sciences:** [**https://www.emsdiasum.com/vials-shell**](https://www.emsdiasum.com/vials-shell)

**I regularly used the 1 dram size (Cat# 72631-10), but get what works for you.**

**This fixation protocol is described in Goeckeritz *et al.* 2023** (please cite this paper if you use this protocol) **(**[**https://journals.ashs.org/jashs/view/journals/jashs/148/2/article-p64.xml**](https://journals.ashs.org/jashs/view/journals/jashs/148/2/article-p64.xml)**) in the Sample Collection part of the methods:**

*“For each date, at least five apices per genotype were collected and bud scales were removed to ensure proper fixation. Then, they were placed in ice-cold fixative (2.5% glutaraldehyde, 2.5% formaldehyde, 0.1 M sodium cacodylate buffer; pH 7.2) and subjected to vacuum infiltration for 30 min at room temperature. Then, fixative was replaced with fresh solution, and at least one additional vacuum infiltration was performed before storing samples at 4 °C in fixative or 0.1 M sodium cacodylate (pH 7.2).”*

**It is recommended you store your samples in fixative to prevent fungal growth – which is extremely rare, but I’ve seen it happen!**

­­­­­­­­­\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

**Equipment**

* Fume hood
* Scale with at least a range of 0 – 500 grams that you can keep in the fume hood
* Vacuum dessicator / chamber (something like this: <https://www.amazon.com/Bel-Art-Polycarbonate-Desiccator-Polypropylene-F42010-0000/dp/B002VBW9R8/ref=sr_1_1_sspa?crid=31LZBIVZPX6IQ&keywords=vacuum%2Bdesiccator%2Bchamber&qid=1706401472&sprefix=vacuum%2Bchamber%2Bdesi%2Caps%2C123&sr=8-1-spons&sp_csd=d2lkZ2V0TmFtZT1zcF9hdGY&th=1>)
* Vacuum pump + tubing to connect pump and chamber
* Something to agitate the samples with – a nutating shaker works. Or you can rig it like I did and tape a container holding the samples onto an orbital shaker (really)
* Oven set to 60°C

**Materials**

* 100% Acetone
* 0.1M sodium cacodylate buffer pH 7.2 (0.4M, Cat #: 11654 Electron Microscopy Sciences)
* Nonenyl succinic anhydride (Cat #: 19051 Electron Microscopy Sciences)
* D.E.R 736 resin (Cat #: 13000 Electron Microscopy Sciences)
* ERL 4221 (Cat #: 15004 Electron Microscopy Sciences)
* DMAE / 2-dimethylamionethanol (Cat #: 13300 Electron Microscopy Sciences)
* Embedding molds (Cat #s: 70900 – 70902 Electron Microscopy Sciences) NOTE: the max width of our diamond knife for sectioning was 8 mm – therefore our sample blocks could not be over this limit!
* Disposable 4 oz specimen containers (for mixing spurr in – I don’t think they necessarily have to be sterile. E.g., these would work: <https://www.globescientific.com/4-oz-specimen-containers-collection-cups.html?psku=5914&cid=0>)
* Disposable Pasteur glass pipettes for handling spurr reagents and to stir it (e.g., Cat #: 70950-12 Electron Microscopy Sciences)
* Pasteur pipette bulbs

\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

**Embedding Protocol Overview –** NOTE, this was written for the specific equipment in the Hollender lab, so some instructions may vary slightly (e.g., other vacuum pumps might not have a silver lever)

Day 1 – washes + dehydrations (ALL DAY for large tissues)

30 min x3 in 0.1M sodium cacodylate buffer pH 7.2

Dehydrations w/ acetone of 30%, 50%, 70%, 80%, 90%, 100%, 100%, 100% overnight

Day 2 – 1:3 spurr::acetone

Day3 – 1:3 spurr::acetone (can cut if rushed)

Day 4 – 1:2 spurr::acetone

Day 5 – 1:1 spurr::acetone

Day 6 – 2:1 spurr::acetone

Day 7 – 3:1 spurr::acetone

Day 8 – 100% spurr

Day 9 – 100% spurr

Day 10 – 100% spurr

Day 11 – 100% spurr

Day 12 – Make Blocks! Place in 60°C oven

Day 13 – Baking…

Day 14 – Baking…

Day 15 – Blocks are ready! 😊

**Day 1**

**Washes & Dehydrations (Note, Day 1 takes roughly 12 hours; so better to start early)**

This is probably the most critical day of the whole protocol. This is because this is where you dry the tissue. If your tissue is small, you might be able to get away with drying things pretty quickly… but the tissues that we fix are huuuuuuge compared to the usual tissues that are fixed via an original protocol given to me by Dr. Alicia Withrow at MSU. So the dehydration steps were extended to all day!  
  
\*Usually we will embed 16 – 20 samples at a time; solution amounts are based on this many samples.

1. **Wash all samples 3 times in 0.1M Sodium Cacodylate Buffer, pH ~7.2, for 30 minutes each.**
2. Wash 1: Empty fixative (glut/form) into waste container. Submerge samples in 0.1M sod cac buffer. Place on top of shaker and let samples shake for at least 30 min.
3. Wash 2: Empty old 0.1M sod cac buffer in all samples, and replace it with new stuff. Shake samples again for 30 min.
4. Wash 3: Repeat b.

During the last shake, you may proceed to step 2.

1. **Make a 30% Acetone solution**. If you have 20 samples, you’ll want to plan for about 120 mL, to have 3 mL per change (you do two changes).

**Equation**:

**M1V1 = M2V2**

M is concentration, and V is the volume of the concentration you want. Let’s say you want to make 120 mL of a 30% Acetone solution. You’ll want to calculate how much of 100% acetone you’ll add to water to make this:

0.3(120) = (1)(X)

X = 36 mL ; So, you’ll mix 36 mL of 100% acetone with 84 mL of dH2O for 120 mL of 30% acetone. You can make this in the disposable cups (At my bench, bottom two drawers on the left)

1. Empty sample containers of the sod cac buffer; submerge samples in glass vials (<https://www.emsdiasum.com/vials-shell>; usually takes 2-3 mL) with 30% acetone solution. Leave caps off.
2. Take samples (with caps off) to vacuum pump. Place samples in vacuum chamber. Put lid on, make sure it is lined up and secure. Be sure the valve connecting tubes is vertical (in line with tubes), not horizontal (perpendicular with tubes). Turn pump on by flicking silver lever.

**Let vacuum for at least 30 min.**

\***Checking the pump is working**: Here it is always good practice to let the vacuum go for a few seconds, turn off the pump, then SLOWLY adjust the valve til you can hear the pressure readjusting. That means your lid is on correctly and everything is functioning properly. Turn the valve vertically again, flick the pump back on.

**When vacuuming is complete, turn off vacuum by flicking silver lever again. *Slowly* twist valve to the horizontal position until you start to hear the pressure adjusting. Once you do, stop twisting the valve and let the pressure readjust (it will stop hissing when it is done)**

c. Empty containers of 30% acetone solution. And submerge samples again with fresh 30% acetone.

d. Put caps on tightly, then put on shaker and shake for 30 minutes.

1. **Make a 50% Acetone solution**. For 20 samples :

(0.5)(120) = 1(X)

X = 60 mL. So 60 mL of 100% acetone, 60 mL of dH20.

1. Empty sample containers of old 30% acetone. Submerge samples in fresh 50% acetone.
2. Take samples (with caps off) to vacuum pump. Place samples in vacuum chamber. Put lid on, make sure it is lined up and secure. Be sure the valve connecting tubes is vertical (in line with tubes), not horizontal (perpendicular with tubes). Turn pump on by flicking silver lever.

**Let vacuum for at least 30 min. Check that pump is working properly.\***

**When vacuuming is complete, turn off vacuum by flicking silver lever again. Slowly twist valve to the horizontal position until you start to hear the pressure adjusting. Once you do, stop twisting the valve and let the pressure readjust (it will stop hissing when it is done)**

1. Empty containers of old 50% acetone solution. Submerge samples again with fresh 50% acetone.

d. Put caps on tightly, then put on shaker and shake for 30 minutes.

1. **Make 70% Acetone solution.** For 20 samples:

(0.7)(120) = 1(X)

X = 84 mL. 84 mL acetone, 36 mL dH2O

1. Empty sample containers of old 50% acetone. Submerge samples in fresh 70% acetone.
2. Take samples (with caps off) to vacuum pump. Place samples in vacuum chamber. Put lid on, make sure it is lined up and secure. Be sure the valve connecting tubes is vertical (in line with tubes), not horizontal (perpendicular with tubes). Turn pump on by flicking silver lever.

**Let vacuum for at least 30 min. Check that pump is working properly.\***

**When vacuuming is complete, turn off vacuum by flicking silver lever again. Slowly twist valve to the horizontal position until you start to hear the pressure adjusting. Once you do, stop twisting the valve and let the pressure readjust (it will stop hissing when it is done)**

1. Empty containers of old 70% acetone solution. Submerge samples again with fresh 70% acetone.
2. Put caps on tightly, then put on shaker and shake for 30 minutes.
3. **Make 80% Acetone solution.** For 20 samples:

(0.8)(120) = 1(X)

X = 96 mL. 96 mL acetone, 24 mL dH2O

1. Empty sample containers of old 70% acetone. Submerge samples in fresh 80% acetone.
2. Take samples (with caps off) to vacuum pump. Place samples in vacuum chamber. Put lid on, make sure it is lined up and secure. Be sure the valve connecting tubes is vertical (in line with tubes), not horizontal (perpendicular with tubes). Turn pump on by flicking silver lever.

**Let vacuum for at least 30 min. Check that pump is working properly.\***

**When vacuuming is complete, turn off vacuum by flicking silver lever again. Slowly twist valve to the horizontal position until you start to hear the pressure adjusting. Once you do, stop twisting the valve and let the pressure readjust (it will stop hissing when it is done)**

1. Empty containers of old 80% acetone solution. Submerge samples again with fresh 80% acetone.
2. Put caps on tightly, then put on shaker and shake for 30 minutes.
3. **Make 90% Acetone solution.** For 20 samples:

(0.9)(120) = 1(X)

X = 108 mL. 108 mL acetone, 12 mL dH2O

1. Empty sample containers of old 80% acetone. Submerge samples in fresh 90% acetone.
2. Take samples (with caps off) to vacuum pump. Place samples in vacuum chamber. Put lid on, make sure it is lined up and secure. Be sure the valve connecting tubes is vertical (in line with tubes), not horizontal (perpendicular with tubes). Turn pump on by flicking silver lever.

**Let vacuum for at least 30 min. Check that pump is working properly.\***

**When vacuuming is complete, turn off vacuum by flicking silver lever again. Slowly twist valve to the horizontal position until you start to hear the pressure adjusting. Once you do, stop twisting the valve and let the pressure readjust (it will stop hissing when it is done)**

1. Empty containers of old 90% acetone solution. Submerge samples again with fresh 90% acetone.
2. Put caps on tightly, then put on shaker and shake for 30 minutes.
3. **100% Acetone step – already made!**
4. Empty sample containers of old 90% acetone. Submerge samples in fresh 100% acetone.
5. Take samples (with caps off) to vacuum pump. Place samples in vacuum chamber. Put lid on, make sure it is lined up and secure. Be sure the valve connecting tubes is vertical (in line with tubes), not horizontal (perpendicular with tubes). Turn pump on by flicking silver lever.

**Let vacuum for at least 30 min. Check that pump is working properly.\***

**When vacuuming is complete, turn off vacuum by flicking silver lever again. Slowly twist valve to the horizontal position until you start to hear the pressure adjusting. Once you do, stop twisting the valve and let the pressure readjust (it will stop hissing when it is done)**

1. Empty containers of old 100% acetone solution. Submerge samples again with fresh 100% acetone.
2. Put caps on tightly, then put on shaker and shake for 30 minutes.
3. **Repeat** **Step** **7.**
4. **Repeat** **Step 7; but for part d., don’t shake for 30 minutes, leave on shaker and shake it overnight. You’re free! Go home! 😊**

**Day 2**

**1 part spurr :: 3 parts 100% acetone**

What the heck is spurr, you ask? Well, it is a substance that slowly polymerizes and forms a hard plastic. That’s what we are doing – ‘embedding’ our samples in a hard plastic. All subsequent days have the goal of slowly replacing all acetone and other stuff inside the cells and filling them with this plastic (spurr). The basic recipe (4 ingredients) is as follows:  
  
Base Spurr Mixture D:

ERL 4221 10g

D.E.R 736 6g

NSA 26g

DMAE 0.2g

**Spurr MUST be made in the hood**. There is a scale in there for that purpose. All ingredients are liquids, and can be found underneath the hood. Once made, **you can store it in the 4C fridge for up to one week.** A 3X batch is all that one of the disposable cups (urine sample cups) will hold, which will give you at least 100 mL. Keep track of how much that you make in your lab notebook, because we will want to record how much we throw into the lab waste containers.

3X Spurr Mixture D:

ERL 4221 30g

D.E.R 736 18g

NSA 78g

DMAE 0.6g

Steps to make 3X spurr batch:

1. Place empty (no lid) disposable cup on scale. **Zero out scale**.
2. **Slowly pour ERL 4221** into disposable cup until it reaches ~30g. **Zero scale again**.
3. **Slowly pour D.E.R. 736** into disposable cup until it reaches ~18g. Notice you can’t overshoot it, or you will now have uneven proportions of stuff. >.< So, make sure you are going slowly! After adding 18g, **Zero out scale again**.
4. **Slowly pour NSA** into disposable cup until it reaches ~78g. Again, don’t overshoot it!! **Zero out scale again**.
5. Get glass disposable pipette (in drawer near centrifuge), and break the tip off of it to the opening is larger (over the broken glass bucket). Get pipette bulb and attach disposable pipette to it. Suck up just a little bit of DMAE, and release it into the disposable cup until it reaches 0.6g.
6. Remove disposable glass pipette from bulb and use it to stir the spurr. Make sure it is very thoroughly mixed (it may take a while). You should see no color variation, and the spurr should be a homogenous golden color. Then throw the disposable glass pipette away in the solid waste.

\*\*Make more spurr as needed, from now on.

1. **Make ~120 mL of 1:3 parts spurr to acetone. (0.25 of spurr, 0.75 100% acetone)**

For 120 mL:

1. / 4 = 30 mL. So 1 part of 120 is 30 mL. So, **30 mL of spurr, 90 mL of acetone. Mix well.** 
   1. Take samples and remove liquid. Submerge samples (~2 mL) with 1:3 spurr:acetone mixture. Leave caps off.
   2. Take samples (with caps off) to vacuum pump. Place samples in vacuum chamber. Put lid on, make sure it is lined up and secure. Be sure the valve connecting tubes is vertical (in line with tubes), not horizontal (perpendicular with tubes). Turn pump on by flicking silver lever.

**Let vacuum for at least 60 min. Check that pump is working properly**.\*

**When vacuuming is complete, turn off vacuum by flicking silver lever again. Slowly twist valve to the horizontal position until you start to hear the pressure adjusting. Once you do, stop twisting the valve and let the pressure readjust (it will stop hissing when it is done)**

* 1. Empty old 1:3 spurr:acetone, and replace it with fresh. Tightly put all caps on and place samples on shaker. Shake for the remainder of the day, til about 4 – 4:30pm.

Towards end of day…

1. **Repeat** **Step** **1. Shake overnight**

**Day 3**

**1 part spurr : 3 parts 100% acetone**

Repeat Day 2. It’s that simple!

**Day 4**

**1 part spurr : 2 parts 100% acetone**

1. **Make ~120 mL of 1 part spurr : 2 parts 100% acetone.**

120 / 3 = 40, so 40 mL is one part of 120. So, **40 mL of spurr to 80 mL of acetone is 1:2 spurr:acetone.**

1. Take samples and remove liquid. Submerge samples (~2 mL) with 1:2 spurr:acetone mixture. Leave caps off.
2. Take samples (with caps off) to vacuum pump. Place samples in vacuum chamber. Put lid on, make sure it is lined up and secure. Be sure the valve connecting tubes is vertical (in line with tubes), not horizontal (perpendicular with tubes). Turn pump on by flicking silver lever.

**Let vacuum for at least 60 min. Check that pump is working properly**.\*

**When vacuuming is complete, turn off vacuum by flicking silver lever again. Slowly twist valve to the horizontal position until you start to hear the pressure adjusting. Once you do, stop twisting the valve and let the pressure readjust (it will stop hissing when it is done).**

1. Empty old 1:2 spurr:acetone, and replace it with fresh. Tightly put all caps on and place samples on shaker. Shake for the remainder of the day, til about 4 – 4:30pm.

Towards end of day…

2. **Repeat** **Step** **1. Shake overnight**

**Day 5**

**1 part spurr : 1 part 100% acetone**

1. **Make ~120 mL of 1 part spurr : 1 part 100% acetone.**

120 / 2 = 60, so 60 mL is one part of 120. So, **60 mL of spurr to 60 mL of acetone is 1:1 spurr:acetone.**

1. Take samples and remove liquid. Submerge samples (~2 mL) with 1:1 spurr:acetone mixture. Leave caps off.
2. Take samples (with caps off) to vacuum pump. Place samples in vacuum chamber. Put lid on, make sure it is lined up and secure. Be sure the valve connecting tubes is vertical (in line with tubes), not horizontal (perpendicular with tubes). Turn pump on by flicking silver lever.

**Let vacuum for at least 60 min. Check that pump is working properly**.\*

**When vacuuming is complete, turn off vacuum by flicking silver lever again. Slowly twist valve to the horizontal position until you start to hear the pressure adjusting. Once you do, stop twisting the valve and let the pressure readjust (it will stop hissing when it is done).**

1. Empty old 1:1 spurr:acetone, and replace it with fresh. Tightly put all caps on and place samples on shaker. Shake for the remainder of the day, til about 4 – 4:30pm.

Towards end of day…

2. **Repeat** **Step** **1. Shake overnight.**

**Day 6**

**2 parts spurr : 1 part 100% acetone**

1. **Make ~120 mL of 2 parts spurr : 1 part 100% acetone.**

120 / 3 = 40, so 40 mL is one part of 120. So, **80 mL of spurr to 40 mL of acetone is 2:1 spurr:acetone.**

1. Take samples and remove liquid. Submerge samples (~2 mL) with 2:1 spurr:acetone mixture. Leave caps off.
2. Take samples (with caps off) to vacuum pump. Place samples in vacuum chamber. Put lid on, make sure it is lined up and secure. Be sure the valve connecting tubes is vertical (in line with tubes), not horizontal (perpendicular with tubes). Turn pump on by flicking silver lever.

**Let vacuum for at least 60 min. Check that pump is working properly**.\*

**When vacuuming is complete, turn off vacuum by flicking silver lever again. Slowly twist valve to the horizontal position until you start to hear the pressure adjusting. Once you do, stop twisting the valve and let the pressure readjust (it will stop hissing when it is done).**

1. Empty old 2:1 spurr:acetone, and replace it with fresh. Tightly put all caps on and place samples on shaker. Shake for the remainder of the day, til about 4 – 4:30pm.

Towards end of day…

2. **Repeat** **Step** **1. Shake overnight.**

**Day 7**

**3 parts spurr : 1 part 100% acetone**

1. **Make ~120 mL of 3 parts spurr : 1 part 100% acetone.**

120 / 4 = 30, so 30 mL is one part of 120. So, **90 mL of spurr to 30 mL of acetone is 3:1 spurr:acetone.**

1. Take samples and remove liquid. Submerge samples (~2 mL) with 2:1 spurr:acetone mixture. Leave caps off.
2. Take samples (with caps off) to vacuum pump. Place samples in vacuum chamber. Put lid on, make sure it is lined up and secure. Be sure the valve connecting tubes is vertical (in line with tubes), not horizontal (perpendicular with tubes). Turn pump on by flicking silver lever.

**Let vacuum for at least 60 min. Check that pump is working properly**.\*

**When vacuuming is complete, turn off vacuum by flicking silver lever again. Slowly twist valve to the horizontal position until you start to hear the pressure adjusting. Once you do, stop twisting the valve and let the pressure readjust (it will stop hissing when it is done).**

1. Empty old 2:1 spurr:acetone, and replace it with fresh. Tightly put all caps on and place samples on shaker. Shake for the remainder of the day, til about 4 – 4:30pm.

Towards end of day…

2. **Repeat** **Step** **1. Shake overnight.**

**Day 8**

**Day 1 of 100% spurr**

1. **Make ~120 mL of spurr.**

1. Take samples and remove liquid. Submerge samples (~2 mL) with spurr. Leave caps off.
2. Take samples (with caps off) to vacuum pump. Place samples in vacuum chamber. Put lid on, make sure it is lined up and secure. Be sure the valve connecting tubes is vertical (in line with tubes), not horizontal (perpendicular with tubes). Turn pump on by flicking silver lever.

**Let vacuum for at least 60 min. Check that pump is working properly**.\*

**When vacuuming is complete, turn off vacuum by flicking silver lever again. Slowly twist valve to the horizontal position until you start to hear the pressure adjusting. Once you do, stop twisting the valve and let the pressure readjust (it will stop hissing when it is done).**

1. Contrary to previous steps, you do *not* need to replace the 100% spurr. Tightly put all caps on and place samples on shaker. Shake for the remainder of the day, til about 4 – 4:30pm.

Towards end of day…

2. **Repeat** **Step** **1. Shake overnight.**

**Day 9**

**Day 2 of 100% spurr**

1. **Repeat Day 8**

**Day 10**

**Day 3 of 100% spurr**

1. **Repeat Day 8**

**Day 11**

**Day 4 of 100% spurr**

1. **Repeat Day 8**

**Day 12**

**Making the blocks! 😊 (I will probably do this step) Do this in the morning, if possible. Otherwise, wait an extra day to take the blocks out (wait til Day 16)**

1. Make 1X batch of FRESH spurr (you don’t need much to make the blocks)
2. Print needed labels or write them with pencil
3. Cut labels out and get block embedding mold. Place the labels in order in the mold wells face down.
4. Put just a little spurr in each mold, using a glass pipette + bulb – just fill halfway. Orient samples in spurr, then gently drop a bit more spurr on the mold with the samples in it to cover the samples completely.
5. If any bubbles are around, use a pipette tip to pop them
6. Take all molds with samples in them to the 60 degree C oven in secondary containment (in a petri dish, for example, in the event the spurr spills over), and shut oven door by closing, and pressing down handle.

**Day 13**

**Wait for it….**

**Day 14**

**Wait for it…………**

**Day 15**

**Blocks are done! Hooray! You made it! 😊 Remove them from the oven and put them on the lab bench to cool. Word of advice – don’t touch them til they’re cool, or you’ll get sticky.**