PART I: Culturing the Cells

Antibiotic stock solutions

Antibiotics are used to in LB Medium and Agar plates to ensure our plasmid is retained within the cell. Store stock concentrations in 500 uL aliquots at -20°C.

Antibiotic	Stock concentration (1000x)	Solvent	Storage	Concentration in culture
Chloramphenicol	170 mg/mL	Ethanol	-20 ℃	170 µg/mL
Ampicillin	100 mg/mL	dH_2O	-20 ℃	100 µg/mL
Kanamycin	50 mg/mL	dH_2O	-20 ℃	50 *µg/mL*

LB media and LB Agar (Plates)

LB is the media for E.coli. There are 2 types of LB made in lab: 1) Liquid LB, and 2) solid LB. The solid LB contains agar and is used to make media plates. Agar is hard to dissolve, do not split the solution after adding agar.

Materials: (to make 500mL)*

- Tryptone (5g)
- NaCl (5g)
- Yeast Extract (2.5g)
- Agar [for PLATES only] (7.5g)
- 500mL milliQ Water

Antibiotics:

• for Ampicillin resistance, add: 500µL of 100 mg/mL stock solution

• for Chloramphenicol resistance, add: 500µL of 35 mg/mL stock solution

Procedure:

1. Add 400 mL of MilliQ Water to conical flask. Use stir bar to keep contents moving.
2. Add tryptone, NaCl, and yeast extract (and agar for plating medium)
3. Adjust pH to 7.0 using KOH or NaOH

4. Bring volume up to 500mL using MilliQ water
5. Autoclave

For LB agar: After autoclaving, add the desired amount of antibiotics **after the LB agar is cooled to ~50-60 °C**. Let the LB agar medium cool to ~55°C before plating.

Pouring plates:

1. Open sleeve of petri plates underneath a sterile fume hood. (500 mL should be sufficient for roughly a sleeve of 25 plates)
2. Take the slightly cooled LB agar solution and pour onto open plates such that the bottom of the plate is covered. (Avoid over-pouring the LB agar! Pour the minimum possible to cover the plate)
3. Allow plates to dry for 10-15 minutes, then invert and store at 4 °C.

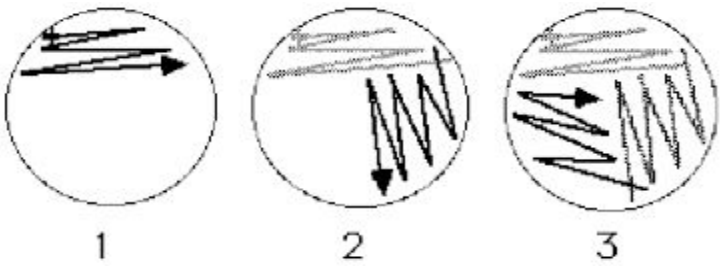
*Note: for larger volumes, increase protocol proportionally

*Plates should generally be used within 2 weeks of preparation.

Streaking Agar Plates

This protocol is adopted from AddGene. Bacteria growing on LB agar plates are for daily and weekly use, and are good up to 4 weeks. If longer storage is required, cells should be frozen.

1. Obtain an LB Agar plate with appropriate antibiotic.
2. Label the bottom of the plate with the plasmid name, date, the antibiotic resistance and your initials.
3. Sterilize the bench by spraying it down with 70% ethanol and wiping it down with a paper towel. Maintain sterility by working near a flame or Bunsen burner.
4. Obtain the appropriate bacterial stab/glycerol stock/ liquid culture.
5. If obtaining bacteria from a bacterial stab or agar plate, use a sterile loop (sterilized by passing over the flame) to touch the bacteria and then streak using technique shown below.



6. If obtaining bacteria from liquid culture, pipette 10 - 100 uL of DNA onto the agar plate, then spread using either a sterile glass spreader (sterilized by spraying with ethanol then flame)or sterile glass beads.
7. After overnight growth, agar plate should be sealed with (plastic or paraffin) and stored upside down (agar side up) to minimize contamination and to keep both the culture and agar properly hydrated.

M9 Medium

M9 is the nitrogen-limiting medium in which our device is grown. The carbon source (either glucose or styrene) varies depending on the experiment.

1. Prepare and autoclave- sterilize 1 M $MgSO_4$ and 1 M $CaCl_2$.
2. If using glucose, filter-sterilize by passing 20% glucose through a 0.22-uM filter

Per liter:

To 750 ml of sterile H_2O (cooled to at least 50°C), add:
5xM9 salts*.....200 mL
1 M $MgSO_4$..... 2 mL
20% of the carbon source..... 20 mL
1 M $CaCl_2$.....2 mL
Sterile deionized water to 980 mL

*5xM9 salts is made by dissolving the following salts in deionized H_2O to a final volume of 1 liter: $Na_2HPO_4 \cdot 7H_2O$ (64 g), KH_2PO_4 (15g), NaCl (2.5g), NH_4Cl (5.0g)

Note: Add $CaCl_2$ before salts to avoid precipitate from forming. Use M9 Medium within 2-3 weeks.

Glycerol stocks

Glycerol stocks can preserve cells for over 5 years. This protocol is adopted from Addgene.

1. Use cells that have been pre-cultured in LB medium overnight.
2. Add .5 mL of the overnight culture to .5 mL of 50% glycerol in a 2 mL in a cryovial and gently mix. Note: Make sure to shake the glycerol before use. Make sure there are no layers present,.
3. Store in -80°C.

4. To recover bacteria from your glycerol stock, open the tube and use a sterile loop, toothpick or pipette tip to scrape some of the frozen bacteria off of the top. **Do not let the glycerol stock unthaw!** Streak the bacteria onto an LB agar plate and grow overnight.

Cell Growth to Produce PHAs

Our PHA producing device is first pre-cultured overnight in LB medium, then washed and transferred to M9 Medium

1. Pre-culture cells
    a. Add 2 mL of LB medium and 2 mL and appropriate antibiotic to culture tubes.
    b. Using a sterilized loop, pick a single colony from an agar plate and transfer cells to the culture tubes by gently shaking the loop in the medium.
    c. Place in shaking incubator for **overnight** (37 °C, 200 rpm)
2. Plate 10 uL of the culture with sterile glass beads or glass spreader on a warmed LB agar plate (with appropriate antibiotic). Check for growth the next day ensure the plasmid is retained.
3. Centrifuge the liquid culture in 1.5 mL sterile eppendorf tubes for 5 min at 5000 rpm.
4. Decant supernatant, add 1 of fresh M9 Medium into each tube, and gently resuspend the pellet.
5. Centrifuge again for 5 min at 5000 rpm, decant the supernatant.
6. Add 1 mL of M9 medium to each tube and gently resuspend the cell pellet again.
7. In 500 mL Erlenmeyer flasks, add 200 mL of M9 medium and the appropriate antibiotic.
8. Add the resuspended cell pellets to the Erlenmeyer flasks.
9. Incubate for **72 hours** (200 rpm, 30 °C) for optimal PHB production.

Styrene Integration

Styrene is integrated into cell medium via partitioning. Styrene is dissolved in Bis(2-ethylhexyl) phthalate before it is added to the medium.

1. In a 500 mL Erlenmeyer flask add, 150 mL of sterile H_2O, 40 mL of M9 Salts, 20 uL of $CaCl_2$, 400 uL of $MgSO_4$, and the appropriate antibiotic.
2. Under the fume hood, add 4 mL of 20% styrene solution (80% DEHP) to the flask.
3. Follow steps 1-6 from *Cell Growth to Produce PHAs*.
4. Add 1 mL of cells (from step 6) to the flask.
5. Incubate for **72 hours** (200 rpm, 30 °C) for optimal PHB production.

PART II: Assembly of Device

Obtaining Backbones from Bacterial Stab

The backbones for our device arrived in bacterial stabs. Upon arrival, store Bacterial Stabs at around 4°C. Bacterial Stabs can be stored for only two weeks in the fridge. For longer-term storage, glycerol stocks should be created.

1. Using a sterile loop, pierce the Bacterial Stab.
2. Store in the fridge overnight. Cell growth will occur at the location of the stab.
3. The next day,

Storing Plasmid Parts

This protocol is for gBlocks Gene Fragments from IDT when they arrive.

1. Centrifuge the tube for 3−5 sec at a minimum of 3000 x g to pellet the material to the bottom of the tube.
2. Add TE to the tube for your desired final concentration
3. Heat block the DNA at 60 °C for 10 minutes. Note: Only do this once.
4. Briefly vortex and centrifuge again.
5. Store at -20°C for up to 24 months.

DNA extraction

This protocol is from the QuickClean II Plasmid Miniprep Kit.

The following steps may be performed ahead of time:

1. Transfer RNase A solution to the Resuspension Buffer and mix well and store at 2-8 °C.
2. Add 100% ethanol (the volume of ethanol to be added is also shown on the bottle labels) to Wash Buffer and mix well.
3. Close the lid immediately after using Lysis Buffer to avoid acidification.
4. The kit can extract high-quality plasmid DNA from 1-5 mL E.coli culture.
5. Adjust the Elution Buffer to the suitable volume if necessary.

Procedure:

1. Transfer 1-1.5 mL E.coli culture to 1.5 mL microcentrifuge tube and centrifuge at 10, 000 rpm (8,000 - 10,000 ×g) for 30 s. Remove and discard the supernatant. Note: this step can be repeated for more than one time to collect enough cells.
2. Add 250 µL Resuspension Buffer to the pellet, cap the tube and resuspend the cells. No cell clumps should be visible after resuspension of the pellets.
3. Add 250 µL Lysis Buffer to the mixture and mix gently by inverting the tube 4-6 times. To avoid contamination by genomic DNA. Do not vortex.
4. Add 350 µL Neutralization Buffer and mix gently by inverting the tube 4-6 times. The solution should become cloudy and no local precipitate should be visible.
5. Centrifuge at 13,000 rpm (>14, 000 ×g) for 10 min until a compact white pellet forms.
6. Transfer the supernatant to the Spin column and centrifuge for 30-60 s at 6,000 ×g. Discard all flow-through.
7. Add 650 µL Wash Buffer to the Spin column and Centrifuge for 30-60 s at 12,000 ×g. Discard the flow-through. Repeat this step once.
8. Centrifuge for additional 1 min at 12,000 ×g to remove residual and transfer the Spin column to a sterile 1.5 mL microcentrifuge tube.
9. Add 50 µL Elution Buffer(ddH2O or TE Buffer) to the Spin column and let the column stand for 1 min at room temperature . The volume of Elution Buffer should be adjusted if necessary.
10. Centrifuge at 12,000 ×g for 1 min. The buffer in the microcentrifuge tube contains the plasmid.
11. Store the DNA at -20°C.

Nanodrop

This protocol is to determine the concentration of DNA within any sample of DNA including miniprep products.

Materials:

- Blanking Solution
- Kimwipes
- Samples
- Nanodrop

Preparation:

Be sure to have proper access to a Nanodrop. This instrument is expensive, but useful.

Procedure:

1. Turn on the computer connected to the Nanodrop

2. Open the Nanodrop program associated with the Nanodrop model
3. Select Nucleic Acid. Machine will run routine wavelength verification, use 1 uL of water for this.
4. Before finding the concentration of the DNA sample, the machine needs a blank. Use the buffer that the DNA sample is in ie. EB buffer or water.
5. Lift arm of the Nanodrop and place 1uL of buffer onto the Nanodrop
6. Set arm back down gently and select Blank.
7. Lift arm, wipe off excess liquid with Kimwipe
8. Place 1uL of DNA sample onto the Nanodrop
9. Set arm back down gently and select Measure
10. Sample concentration appears as ng/uL

Restriction Digest

Based off restriction enzymes and buffers from New England Biolabs.

Note: If this doesn't work well for cloning or gels keep having uncut bands, consider the following from Addgene: Diagnostic digests typically involve ~500 ng of DNA, while molecular cloning often requires 1-3 µg of DNA. For digests with >1 µg of DNA used for cloning, it is recommended that you digest for at least 4 hours.

Procedure:

1. Decide what buffer to use based on the enzymes you are using for your digest, use the following website:
    a. https://nebcloner.neb.com/#!/redigest
2. For 50 uL Reaction
    a. 1 ug of DNA (use less DNA if DNA is scarce) (gels: 200 ng of DNA should be okay!)
    b. 5uL of respective NEB Buffer (10X)
    c. 1uL of enzyme 1
    d. 1uL of enzyme 2
    e. Remaining should be MilliQ Water
3. In rxn tube, mix the following order: **1. Water; 2. Buffer; 3. DNA; 4. Enzymes**
4. Incubate at 37C for 30 minutes (If you have time save enzymes otherwise incubate for 1hr) then heat kill at 80C for 20 mins
    a. Note: heat kill temperature will depend on restriction enzymes, check the enzyme heat kill temperature.

Gibson Assembly

We first attempted to construct our device using the Gibson Assembly method.

Procedure:

1. Gather the required materials on ice: DNA fragments for assembly (already resuspended), linearized vector (amp/camR backbones), GenBuilder 2x Master Mix, DI H2O
2. In a tube, mix the correct amount of each DNA fragment, the GenBuilder 2x Master Mix and DI H2O. Note: if the total volume is exceeded, use a 1:1 ratio of Master Mix to DNA.
3. Gently mix the reaction by pipetting.
4. Incubate the reaction in a thermocycler at 50°C for 60 minutes
5. For electroporation, dilute the reaction product 5-fold and use 1 µl for transformation.
6. Concentrate the cells by centrifuging (10 min, 800 g) removing the supernatant, and resuspending in uL of fresh LB medium. Plate 1/10 of the volume (~20 uL with just PHA plasmid) of the concentrated cells on selection plates. For a positive control reaction, spread 1/10 volume of the cells on LB Agar plates containing 100 mg/ml ampicillin and 0.1 mM IPTG.
7. Incubate the plates overnight at 37°C. A successful positive control reaction should produce hundreds of red colonies on the selective plates.

Ligation Assembly

We also attempted to assemble our plasmids using ligation assembly.

1. Follow an agarose gel electrophoresis procedure, and run DNA fragments on an 0.8% agarose gel.
2. After the gel has run, move it to an open UV box. **Be sure to wear proper UV protection**.
3. With a clean, sterile razor blade, slice the desired DNA fragments from the gel. Try to minimize DNA exposure to UV light to prevent dimer formation. Put each fragment into separate, sterile eppendorf tubes.
4. Melt the agarose fragments by placing them in a 65°C water bath for 10 minutes. Hold them at room temperature.
5. In a sterile eppendorf tube, prepare a 20 uL ligation reaction
    a. 2 uL of T4 DNA Ligase Buffer (10X)
    b. 10 uL total of agarose DNA
    c. 7 uL MillQ Water
    d. 1 uL of T4 Ligase

*add water, buffer, vector, insert, then ligase

6. Ligate for a total of 3 hours at room temperature, adding a the first insert at the beginning, the second insert after 30 minutes, and all following inserts in 15 minute increments. Start with the vector.
7. Transform using 2-4 uL of ligation reaction.

Golden Gate Assembly

Our final device was constructed using Golden Gate assembly. Adapted and optimized from NEB Golden Gate Assembly for BsaI Type II restriction site .

Materials:

- NEB Golden Gate Buffer
- NEB Golden Gate Master Mix
- Golden Gate Primers

*Before starting, make sure DNA fragments do not have BsaI restriction sites. If there are BsaI sites, perform site directed mutagenesis or order parts without the type II restriction site

Designing Golden Gate Primers:

Use NEB Golden Gate Assembly Tool

- https://goldengate.neb.com/editor
- When ordering primers, adjust temperatures to match the polymerase you are going to be using for PCR (NEB Q5 has fairly unique melting temperatures)
- Save benchling NEB tool outputs

Procedure:

1. Run PCR reaction on all the parts to add golden primers onto the parts (the BsaI restriction sites are added in this step)
    a. When setting the annealing temperatures, only half of the primer must anneal. The part that anneals is the part that is unique to each primer. Generally the lower case letters outputted in the benchling pdf from the tool
    b. To find the proper annealing temperatures for this part of the primer, use the NEB TM calculator (https://tmcalculator.neb.com/#!/main) optimized for whatever polymerase you are using
2. If the part you are inserting is inside a plasmid that is same antibiotic resistance as your final destination vector, you should gel extract the linear part with after the pcr reaction
3. PCR purify all pcr products
4. Set Up Golden Reaction on ice:
    a. 75-100 ng of Destination Vector
    b. 2:1 Molar Ratio (insert:vector)

        i. Use neb molar ratio calculator: https://nebiocalculator.neb.com/#!/ligation
    c. 2 uL of NEB Golden Gate Buffer (10X)
    d. 1 uL NEB Golden Gate Assembly Mix
    e. Up to 20 uL of MilliQ Water
        i. Add water first, then Buffer, DNA/Vector, and lastly Assembly Mix
5. Incubate reaction in thermocycler:
    a. For 1-4 inserts: 37C 1hr → 55C 5min
    b. For 5-10 inserts: (37C 1min → 16C 1min) x 30 → 55C 5min
    c. For 5-10 inserts: (37C 5min → 16C 5min) x 30 → 55C 5min
6. Transform 2-4 uL of reaction product

Preparing Electrocompetent Cells

In order to transform our assembled plasmids into E. coli cells, they were made electrocompetent using this procedure.

1. Grow 400ml of E. coli culture to OD600=0.5-0.7 (600nm)
2. Leave on ice for 15-30 mins
3. Pellet cells at 4C for 15 mins at 4000g
4. Decant supernatant and resuspend in pellet in 400 mL ice-cold 10% sterile glycerol
5. Spin again as before and resuspend in 10mL 10% sterile glycerol
6. Spin again as before and resuspend in 0.8ml 10% sterile glycerol
7. Make 50 ul aliquots of cell suspension → work on ice
8. Freeze aliquots in liquid nitrogen and store at -80C

Electroporation

Our plasmids are transformed into cells using electroporation. Electroporation uses high-voltage electric shocks to introduce DNA into cells.

Notes:

- Heat ligation products for 70 ºC for 15 minutes
- Pre-chill microcentrifuge tubes and electroporation cuvettes on ice

1. Have Recovery Medium and sterile culture tubes readily available at room temperature (one tube for each transformation reaction). Transformation efficiency may decrease with the use of SOC or other media.
2. Place electroporation cuvettes (0.1 cm gap) and microcentrifuge tubes on ice (one cuvette and one microfuge tube for each transformation reaction).

3. Remove Cells from the -80 °C freezer and thaw completely (10-15 minutes).
4. When the cells are thawed, mix them by tapping gently. Aliquot 25 µL of cells into a chilled microcentrifuge tubes on ice.
5. Add 1 µl of 5x diluted DNA to the chilled microcentrifuge tube.
6. Carefully pipet 25 µL of the cell/DNA mixture into a chilled electroporation cuvette without introducing bubbles. Tap down the cell/DNA mixture to the bottom of the cuvette
7. Within 10 s of the pulse, add 1 mL of Recovery Medium to the cuvette and pipet up and down three times to resuspend the cells. Transfer the cells and Recovery Medium to a culture tube.
8. Place the tube in a shaking incubator at 250 rpm for 1 hour at 37 °C.
9. Spread up to 100 µL of transformed cells on LB agar plates containing the appropriate antibiotic.
10. Incubate the plates overnight at 37 °C. Transformed clones can be further grown in TB or in any other rich culture medium

DNA Sequencing

To confirm that our device assembled properly, it is sequenced using GeneWiz technology.

1. For orders with <48 samples, use 8-strip PCR tubes to streamline preparation and processing. Label your tubes on the side with your initials and sample number. For orders with ≥48 samples, you can receive a discount by using a 96-well PCR plate and arranging the samples vertically (A1 to H1)
2. Dilute your sequencing primer to 5 µM (pmol/µl) using water. You will need 5 µl for each sequencing reaction. Remember that only one primer is used in a sequencing reaction.
3. For the amount of template needed in each of our DNA Sequencing Services (Pre-Mixed, Pre-Defined, and Custom), please refer to the tables below.
4. Prepare template in 10 µl for each sequencing reaction. Please make dilutions in water or Tris. For best results, do not use Tris-EDTA (TE) because EDTA will inhibit the sequencing reaction

DNA Type	DNA Length (include vector)	Template Concentration in 10 µl	Template Total Mass	Your Primer Total Picomoles	Premixed Volume* (Template + Your Primer)
Plasmids	<6 kb	~50 ng / µl	~500 ng	25 pmol	15 µl
	6 - 10 kb	~80 ng / µl	~800 ng		
	> 10 kb	~100 ng / µl	~1000 ng		
Purified PCR Products	<500 bp	~1 ng / µl	~10 ng	25 pmol	15 µl
	500 - 1000 bp	~2 ng / µl	~20 ng		
	1000 - 2000 bp	~4 ng / µl	~40 ng		
	2000 - 4000 bp	~6 ng / µl	~60 ng		
	> 4000 bp	Treat as plasmid	Treat as plasmid		

*If you use a GENEWIZ Universal Primer, submit the required amount of template in 10 µl.

PART III: Extraction/Analysis

Lyophilization

Used when PHB producing cells need to be preserved.

1. Centrifuge down 200mL of cells into a pellet (5000 rpm, 20 min)
2. Discard the supernatant, resuspend the cells into 20 mL of fresh M9 medium, transfer cells to 50mL falcon tubes, and centrifuge again (5000 rpm, 10 min)
3. Discard the supernatant
4. Poke holes with hot steel rod onto the lid of the 50mL falcon tubes
5. Place tubes with cell pellet into liquid nitrogen for ~5 min or until pellets are frozen
6. Take falcon tubes and place them inside of lyophilizer cylinder and attach to lyophilizer
7. Allow cells to dry overnight

SDS/Chloroform Extraction

PHBs are commonly extracted using SDS, then later purified by dissolving in chloroform and precipitating in methanol.

1. Add SDS powder to 200 mL cell culture according to cell dry weight: SDS weight ratio of 0.1-0.7
2. Allow the culture/SDS solution to react for one hour in shaking incubator (200 rpm, 30°C)
3. Heat treat the solution at 121°C for 15 min
4. Centrifuge the heat treated solution (13000 x g , 10 min)
5. Remove supernatant, add 30 mL of distilled water to centrifuge tube and vortex until pellet is fully resuspended.
6. Dry solution at 60°C for 5 h to recover pure P3HB
7. Follow up with dissolving P3HB in chloroform for 24 hours, filtering, then precipitating the filtered solution in ice-cold methanol (70 mL cold methanol : 30 mL water.).

Sonication and Separation of PHB by Density

Eventually, we were able to develop our own unique extraction method. This method prioritizes minimizing the environmental impact of extraction over product purity.

1. Transfer cell culture into a 500 mL centrifuge bottle.
2. Centrifuge at 5000 for 20 min.
3. Decant the supernatant and resuspend the cell pellet in ~20mL of Milli-Q water. Pipette the resuspended cells into a sonication tube.
4. Sonicate cells 5-7 times, or until about 80% of the cells are lysed (observe cells under microscope).
5. Use flat tip
    a. 5-7x sonication for 30 with 15 sec in between
    b. 70-80% amplitude
6. Prepare a sucrose solution with a density of 1.2g/mL.
7. Add sucrose solution to the sonicated cells.
8. Centrifuge the solution at 6500 rpm for 15 min at 6° C.
9. Two pellets should be visible, the bottom pellet contains PHBs.
10. Decant the supernatant and remove the top (cell) pellet from the tube.
11. Resuspend the remaining PHB pellet in water (vortex if necessary)
12. Filter and solution and allow PHBs dry overnight.

Red Nile Dye

Red Nile was used as an assay for PHB production.

1. Prepare samples:
    a. Centrifuge down 1mL of PHA cultures in eppendorf tubes, discard the supernatant
    b. Resuspend the cell pellet in the remaining ~30-50 µL medium that congregates from the tube walls within ~1 min to create a "sludge"
    c. In separate eppendorf tubes, add 2 µL of diluted Nile Red solution to 12µL of cells
2. Prepare agar pads +/- controls
    a. Pipette 100 µl of hot (~60 °C) agarose solution (1% [w/v] in H_2O) on the slide and immediately place the cover slip on the agarose.
    b. Let agarose solidify for ~2 min and carefully remove coverslip with a blade
3. Drop 2µL of Nile Red/cell culture solution onto the agarose pad within 1 min of removing the cover slip and immediately cover again
4. Examine sample under fluorescence microscope (564/40 nm; emission 594)

TEM

**This protocol is a modification of a protocol developed by Jiamin Tian in the article: https://jb.asm.org/content/187/11/3814*

Performed in Lab:

1) Before starting procedure:
   a. 4 days in advance, start a 4 mL preculture with a single colony of the desired bacteria.
   b. After 24 hrs, transfer the bacteria to a 200 mL culture and culture for 72 hrs at 30 degrees celsius at 200 RPM.

2) Pre-fix and fix of the culture
   a. Add 5 mL of the cell culture to a 15 mL falcon tube containing 5 mL of the fixation solution (2% [vol/vol] glutaraldehyde, 3% [wt/vol] paraformaldehyde made fresh, 5% [wt/vol] sucrose, and 0.1 M sodium cacodylate buffer, pH 7.4)
   b. Mix manually by inverting for 5 minutes and then spin the cells down at 5000 rpm for 10 min
   c. Remove the supernatant and resuspend the cells in 10 mL of fresh fixation solution and incubate at room temperature for 1h with occasional manual mixing
   d. Spin down the cells again (5000 RPM for 10 min) and remove the supernatant. Add 1.5 mL of 0.1 M Sodium Cacodylate buffer (7.4 pH) and transfer to an eppendorf tube and mixed for two minutes.
   e. Spin down the cells at 9,000 rpm for 1 minute
   f. Decant fixative and wash the cell pellet three times with 1.5 mL of Sodium Cacodylate buffer (7.4 pH)

3) Osmium Fixation
   a. Fix the cell pellet 1.5 mL of a 1% osmium solution (1.25 mL 4% OsO_4, 1 mL 0.1 M HCl, 1.75 mL dH_2O, and 1 mL acetate-Veronal stock (1.2% [wt/vol] anhydrous sodium acetate and 2.9% [wt/vol] sodium barbiturate [Veronal] in distilled H2O))
   b. Dislodge pellet from bottom of tube and incubate for 1h at room temperature in the osmium solution.
   c. Pellet the cells and decant the supernatant and wash briefly (<1 min) with 1.5 mL of the third fixative solution, Kellenberger uranyl acetate solution (0.5% [wt/vol] uranyl acetate in Veronal-acetate buffer)
   d. Fully resuspend with 1.5 mL of additional fresh Kellenberger uranyl acetate solution and incubate overnight in the dark.

4) Dehydration

a. After uranyl acetate staining, dislodge and rinse the cell pellet quickly with ~1.5 mL of dH_2O, then spin down the pellet.
b. Place the pellet in 50% [v/v] ethanol for 10 minutes then transfer to 70% [v/v] ethanol for 10 minutes, then transfer to 95% [v/v] ethanol for 10 minutes, and finally to 100% ethanol 3 times for 15 minutes. (Make sure to spin down pellet after each round and dislodge before adding the next solution!)
c. Further dehydrate the pellet in a solution of 50% (vol/vol) ethanol-50% (vol/vol) propylene oxide for less than 5 minutes and then transfer to 1.5 mL 100% propylene oxide for 5 minutes.
d. Spin down and decant supernatant, then place the pellet in 50% propylene oxide-50% low-viscosity embedding resin* and rotate on a rotator for 12h.

*Embedding resin is made with the Spurr kit

5) Embedding

a. Transfer pellet into ~1.5 mL low-viscosity embedding resin and place under a vacuum in a desiccator for 4h.
b. Repeat the process a minimum of three times with fresh embedding resin
c. Cut the pellet into small pieces and place them in beam capsules containing 100% low-viscosity embedding resin.
d. Let the beam capsules embed overnight at 60 °C

6) Sectioning and Microscopy

a. Slice ~70 nm sections with a diamond knife
b. Pick up sections 200-mesh nickel grids coated with Formvar (0.3% [wt/vol] dissolved in ethylene dichloride) and a layer of carbon.
c. Examine the sections on an Electron Microscope at 80 kV.

Solutions:

1) Fixation solution
    a) 2% [vol/vol] glutaraldehyde
    b) 3% [wt/vol] paraformaldehyde made fresh
    c) 5% [wt/vol] sucrose
    d) 0.1 M sodium cacodylate buffer (pH 7.4)
2) Sodium Cacodylate buffer (pH 7.4)
    a) ?
3) Osmium solution (1%)

a) 1.25 mL 4% OsO_4
b) 1 mL 0.1 M HCl
c) 1.75 mL dH_2O
d) 1 mL acetate-Veronal stock

4) Acetate-Veronal stock
    a) 1.2% [wt/vol] anhydrous sodium acetate
    b) 2.9% [wt/vol] sodium barbiturate [Veronal] in distilled H2O
5) Kellenberger uranyl acetate solution
    a) 0.5% [wt/vol] uranyl acetate in Veronal-acetate buffer