Prepare Fix:

* 2% PFA/ 2% glut in 0.1M phosphate buffer, pH 6.8

\*higher glut may decrease immunoreactivity with variation between antibodies

Vibratome:

* Cut sections for freeze substitution at 100-200um

Pretreatment (for improved structure):

* 3x10minutes 0.1M Sodium Acetate (NaA)
* 1hr 0.1% CaCl2/NaA
* 3x 5minutes NaA

Cryoprotection (may not be necessary for HPF):

* 30minutes 10% glycerol/NaA
* 30minutes 20% glycerol/NaA
* 30minutes 30% glycerol/NaA
* Overnight 30% glycerol/NaA

AFS Prep:

* Preclean flo-through capsules and aclar spears and dry overnight in oven @ 60C
* Make 2% UA in methanol (~5ml per capsule holder), store in refrigerator and filter before use

AFS:

* Chill chamber, reagents, and tools to **-90C**
* Methanol rinse 2x10minutes
* 2% UA/methanol
* **Set program to run @ -90C for 32 hours**
* **Increase temp +4C/hr for 11.3 hours to -45C**, still in UA/methanol
* **-45C for 50 hours**

Lowicryl HM-20 (need minimum of 5ml fluid per metal can per step)

Crosslinker D 2.98g 2.24g 1.49g

Monomer E 17.02g 12.76g 8.51g

**MIX GENTLY.**

**ADD/MIX GENTLY:**

Initiator C 0.1g 0.075g 0.05g

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**20ml 15ml 10ml**

* + Methanol 3x15minutes
  + 1:1 lowicryl/methanol 2 hours
  + 2:1 lowicryl/methanol 2 hours
  + Lowicryl 2 hours
  + Lowicryl overnight
  + Dry gel caps, spider caps, containers, and tools overnight in oven @ 60C
  + Next day, set up curing capsules with fresh lowicryl, spider-cap, tissue capsules. Transfer all with spider capsules to chilled ethanol bath (15ml). Start UV and complete the -45C for 50 hours step.
* **+5C/hour for 9 hours (up to 0C), still with UV lamp on**
* **0C for 40 hours with UV on**
* Remove from AFS and ethanol bath. Label spiders. Cure capsule-up in CO2 chilled UV box in hood overnight with UV