Optimized rapid high pressure freezing of mouse tissue for the preservation of physiological extracellular space

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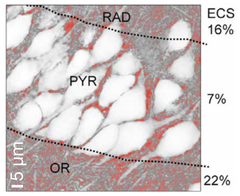
Summary

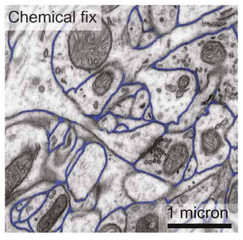
- Extracellular space (ECS) is critical for cell-to-cell cooperation and communication—essential for complex tissue function
- Preservation of ECS can be achieved with specialized preparation methods
- High-pressure freezing (HPF) can preserve tissue ECS, but methodologies vary and procedures are practically difficult
- Here, we present an accessible biopsy HPF method, and demonstrate ECS preservation across multiple mouse tissues

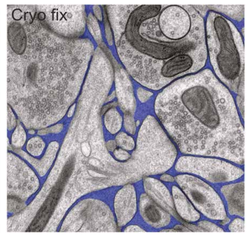
Brain extracellular space volume previously measured to be ~20%

- STED imaging of acute brain slices with cell-impermeable dye
  - Tønnesen J., ..., Nägerl U.V. (2018)
  - Dembitskaya Y., ..., Nägerl U.V. (2023)
- Diffusion measurements: Nicholson, C. & Phillips, J. M. (1981)



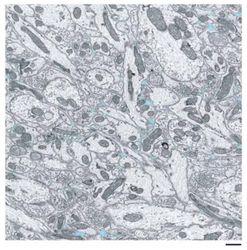
Prior efforts to preserve extracellular space

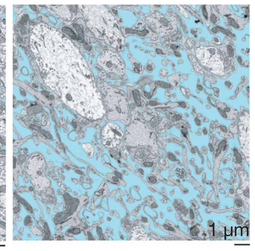




Rapid cryopreservation (slam freezing, high-pressure freezing)

- Van Harreveld A., ..., Malhotra S.K. (1965)
- Korogod N., ..., Knott G.W. (2015)
- Huang X., ..., Han H. (2023)





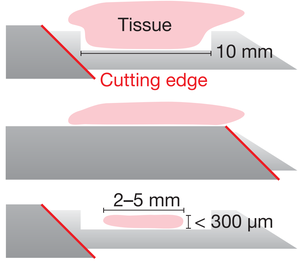
Optimization of buffer osmolarity

- Cragg B. (1980)
- Mikula S. & Denk W. (2015)
- Pallotto M., ..., Briggman K.L. (2015)
- Lu, X., ..., Lichtman J.W. (2023)

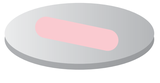
- Cryopreservation is practically difficult, ECS analysis only performed on brain
- Buffer osmolarity optimization: ECS swelling, loss of protein density in ECS

Biopsy high-pressure freezing (HPF) method

Commercially available biopsy needles, 20 or 22 gauge
Argon Medical SuperCore, Merit Medical TEMNO



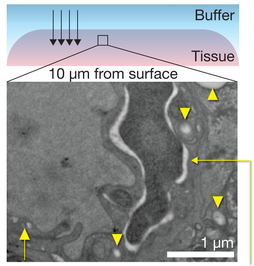
3 mm diameter 150 µm depth aluminum planchette filled with 1-hexadecene



- 8 wk/old C57BL/6 mouse deeply anesthetized with ketamine/xylazine
- Tissue frozen in 50–60 seconds from biopsy
- Subsequent biopsies taken for up to 5 min
- Freeze substitution w/ 0.1% UA, 1% OsO_4, 1% glutaraldehyde, 2% H_2O in acetone
- Infiltrated and embedded in Durcupan

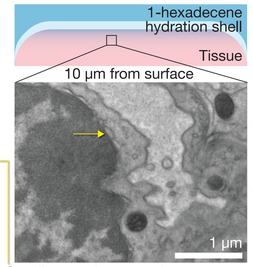
1-hexadecene better preserves tissue physiology

22% BSA: High osmotic pressure, altered pH



Swollen endoplasmic reticulum

1-hexadecene: hydration shell of native extracellular fluid



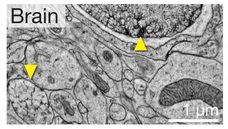
Swollen nuclear envelope

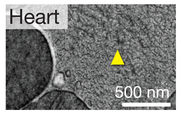
- Aqueous cryoprotectants with altered osmolarity induce tissue stress 30–50 µm from surface
- Stress phenotypes seen in brain, heart, liver, and kidney
- 1-hexadecene (non-aqueous) preserves a native hydration shell, improving physiology

Streamlined screening for well-vitrified FIB-SEM-scale volumes

HPF sample embedded in Durcupan between Aclar sheets

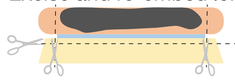
Superglue mount on block, TEM screening of surface





- Scan for segregation patterns indicating ice damage (arrowheads)
- If none, move on

Excise and re-embed longitudinally

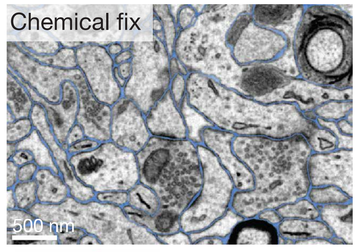


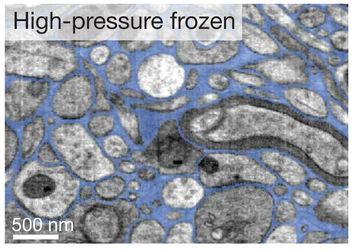
TEM to assess vitrification

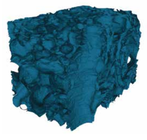
	Screened	Candidates	Success rate
Brain	56	3	5%
Heart	20	1	5%
Liver	17	3	17%
Kidney	24	1	4%
Lung	13	1	7%

- Some tissues vitrify better than others, liver seems to freeze the best
- Which organelles display ice damage first depends on the tissue

Pilot HPF+FIB-SEM run recapitulates prior ECS findings





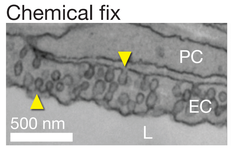


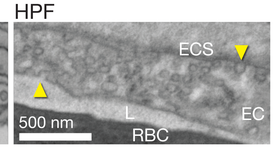
ECS mesh

Manual extracellular space segmentation of 46.1 µm³ volume yields 22.27% ECS by volume, in agreement with prior measurements from literature

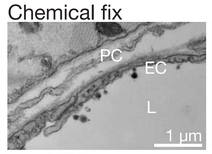
Tissue morphologies in conventional chemical fixation and high-pressure freezing

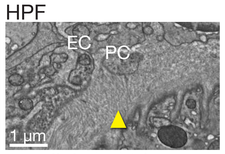
Heart (left ventricle) vasculature





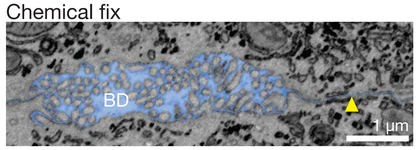
HPF has less docked caveolae (arrowheads), wider caveolae diaphragms. L = vessel lumen, EC = endothelial cell, PC = pericyte, RBC = red blood cell

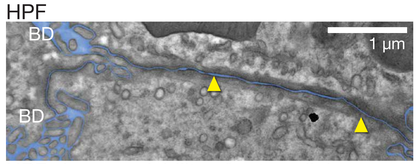




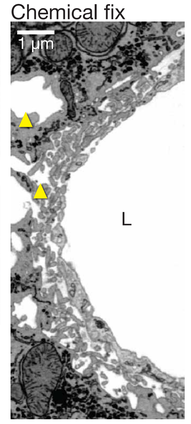
HPF preserves extracellular matrix proteins, does not shrink perivascular cells. Arrowhead = collagen bundles

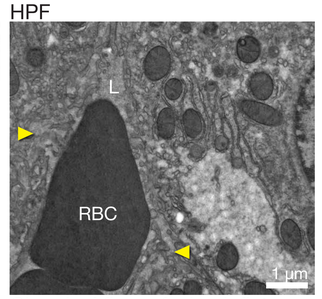
Liver





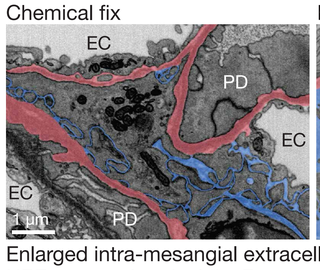
HPF shows variability of extracellular space (blue overlay) between neighboring hepatocytes (arrowheads). BD = bile duct

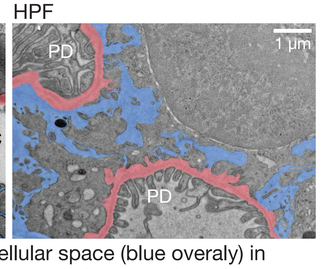




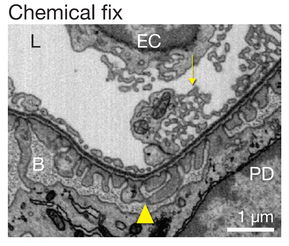
Chemical fix perfusion inflates the blood vessel lumen and space of Disse (arrowheads)

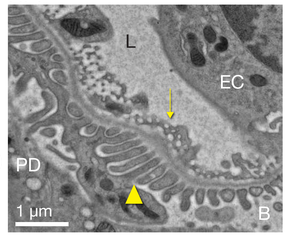
Kidney glomerulus





Enlarged intra-mesangial extracellular space (blue overaly) in HPF versus chemical fix. Basement membrane (red overlay) largely looks unchanged. PD = podocyte end feet (pedicels), EC = endothelial cell





HPF generally has rounder endothelial cell fenestrae (arrow) and podocyte end feet (pedicels, arrowheads). Bowman's space (B) and vessel inflation looks largely unchanged

Future Directions

- Acquire 8 nm voxel size, 100x100x100 µm³ FIB-SEM volume for brain, heart, liver, kidney, and lung
- Manual ECS annotations of paired chemically-fixed and HPF FIB-SEM volumes
- ECS segmentation model training + inference
- Quantitative comparisons of ECS volume, topology, and ECM architecture

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References

[1] Tønnesen et al., *Cell* (2018) [2] Dembitskaya et al., *Nat. Commun.* (2023), [3] Nicholson and Phillips, *J. Physiol.* (1981), [4] Van Harreveld & Malhotra, *J. Cell Biol.* (1965), [5] Korogod et al., *eLife* (2015), [6] Huang et al., *Sens. Imaging* (2023), [7] Cragg, *Tissue and Cell* (1980), [8] Mikula and Denk, *Nat. Methods* (2015), [9] Pallotto et al., *eLife* (2015), [10], Lu et al., *Cell Rep. Methods* (2023).
Prior biopsy needle protocols: Hohenberg et al., *J Microsc.* (1996), Vanhecke et al., *J Microsc.* (2003)