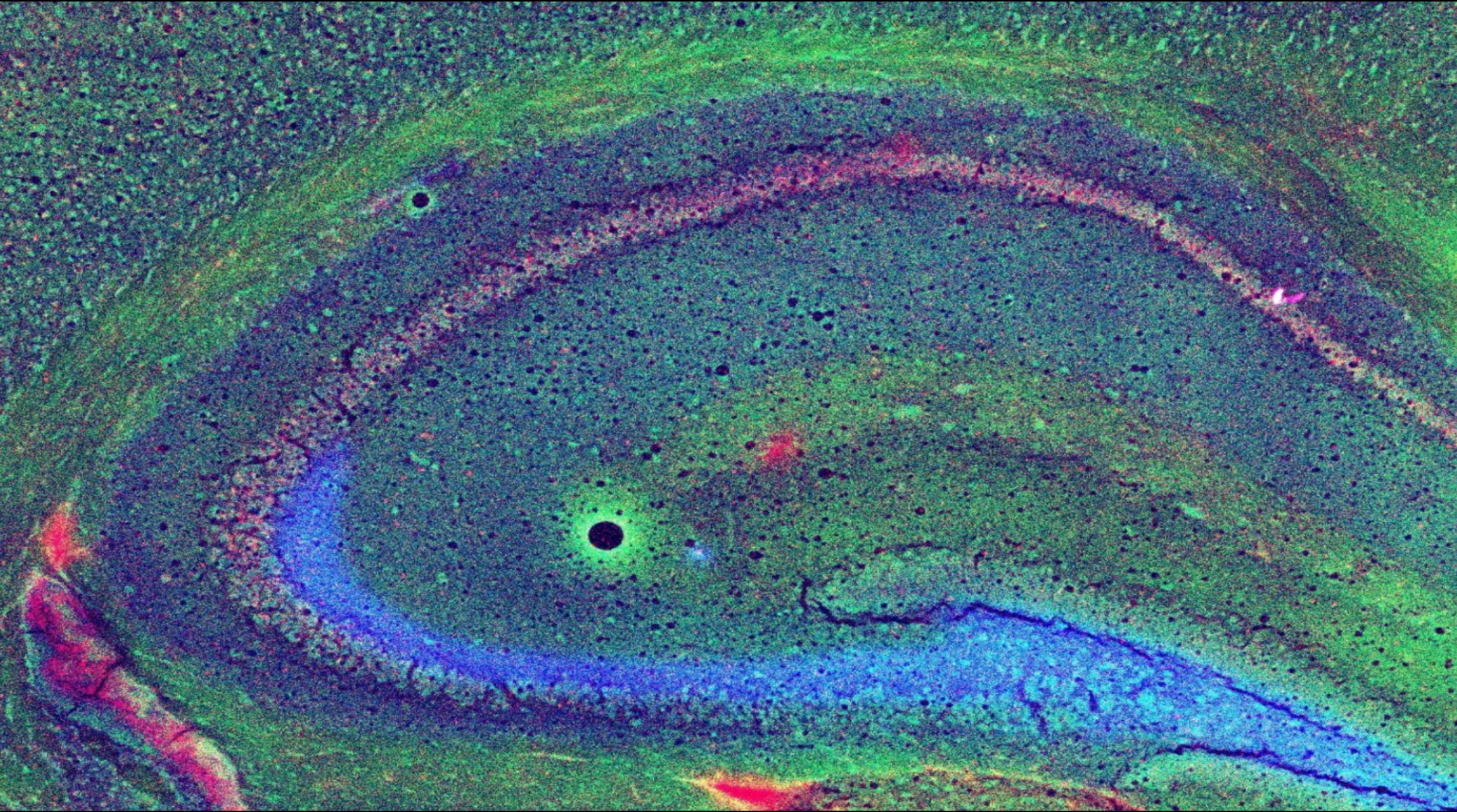
Sample Preparation Considerations for Multi-Modal IRM and XFM Analyses of Biological Tissues



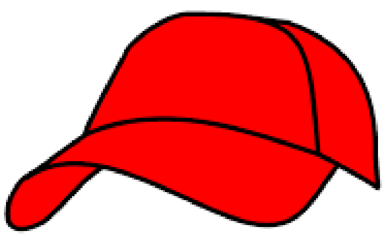
IRM & XFM Workshop, May 20th 2021

Dr Mark Hackett – Curtin University

Curtin University

BACKGROUND

- BSc (Hons) Analytical Chemistry (Curtin University, 2006)
- PhD Chemistry (The University of Sydney, 2007 - 2010)
  - *Spectroscopic investigation of cerebral malaria*
    - *ischemia and blood-brain-barrier*
- Research Fellowships in Canada (University of Saskatchewan 2011 - 2015)
  - *Spectroscopic studies of neurodegeneration after stroke*
    - *ischemic stroke, intra-cerebral haemorrhage*
- Senior Lecturer in Analytical Chemistry at Curtin University (2016)
  - *Developing and applying spectroscopic approaches to study chemical mechanisms of memory loss during ageing and dementia*



Analytical Chemistry
Applied Spectroscopy

Neuroscience
Neurochemistry

CURTIN HEALTH INNOVATION RESEARCH INSTITUTE

Curtin University

dementia australia

Australian Government
Australian Research Council

ANSTO

CHEMISTRY @ CURTIN

CURTIN HEALTH INNOVATION RESEARCH INSTITUTE

Curtin University

TALK OUTLINE

- Themes
  - Techniques
  - Sample Preparation
  - Combined Histology or Immuno-histochemistry with XFM or IRM
- Research Examples
  1. Colocalisation of metal ions with macromolecules in amyloid plaques
  2. Brain-Fe homeostasis during natural ageing
  3. XANES imaging to study hippocampal Zn speciation
  4. Medium energy XAS to study sulfur speciation after stroke

ANSTO
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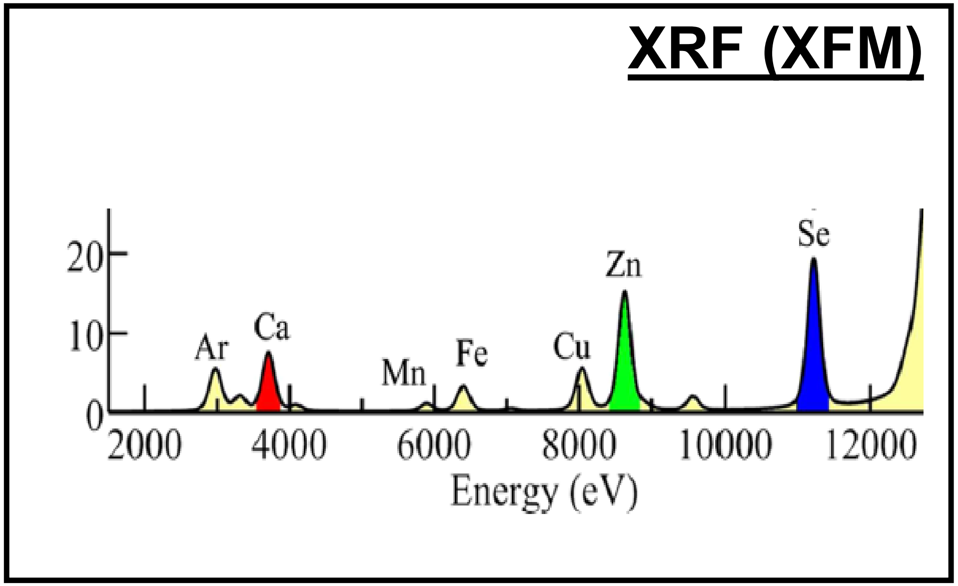
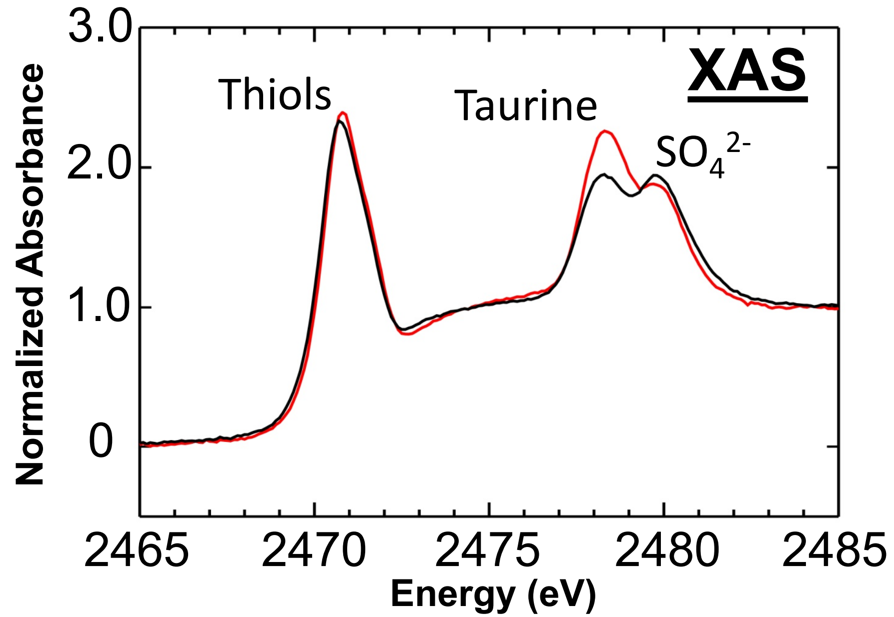
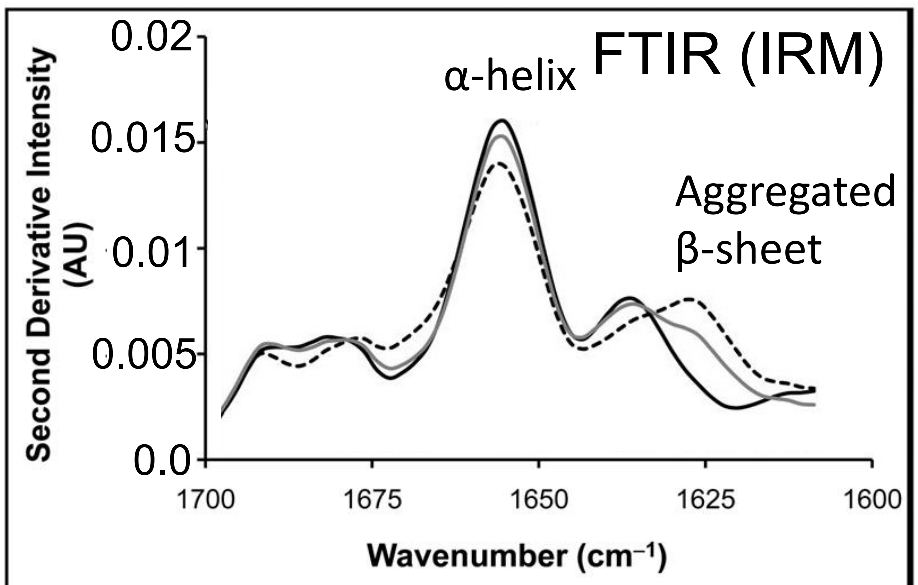
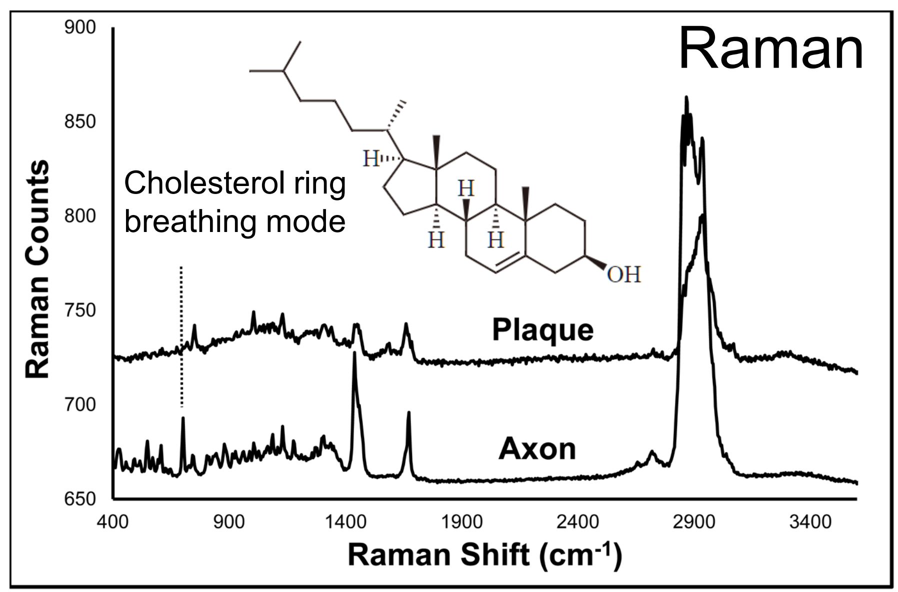
Australian Research Council

INTRODUCTION: THE TECHNIQUES I USE…….







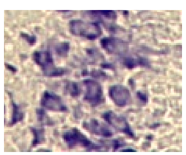


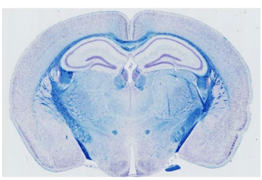
WHAT IS THE ROLE OF SPECTROSCOPIC IMAGING?

Morphology
(**WHERE**)

Chemical / Composition
(**WHAT/HOW MUCH**)

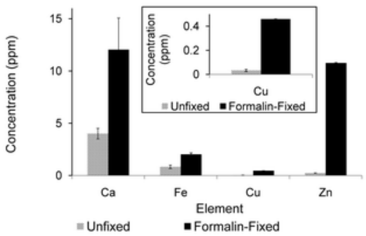
Light Microscopy
Electron Microscopy





IRM, XFM, XAS, Raman
LA-ICP-MS, TOF-SIMS, PIXE……

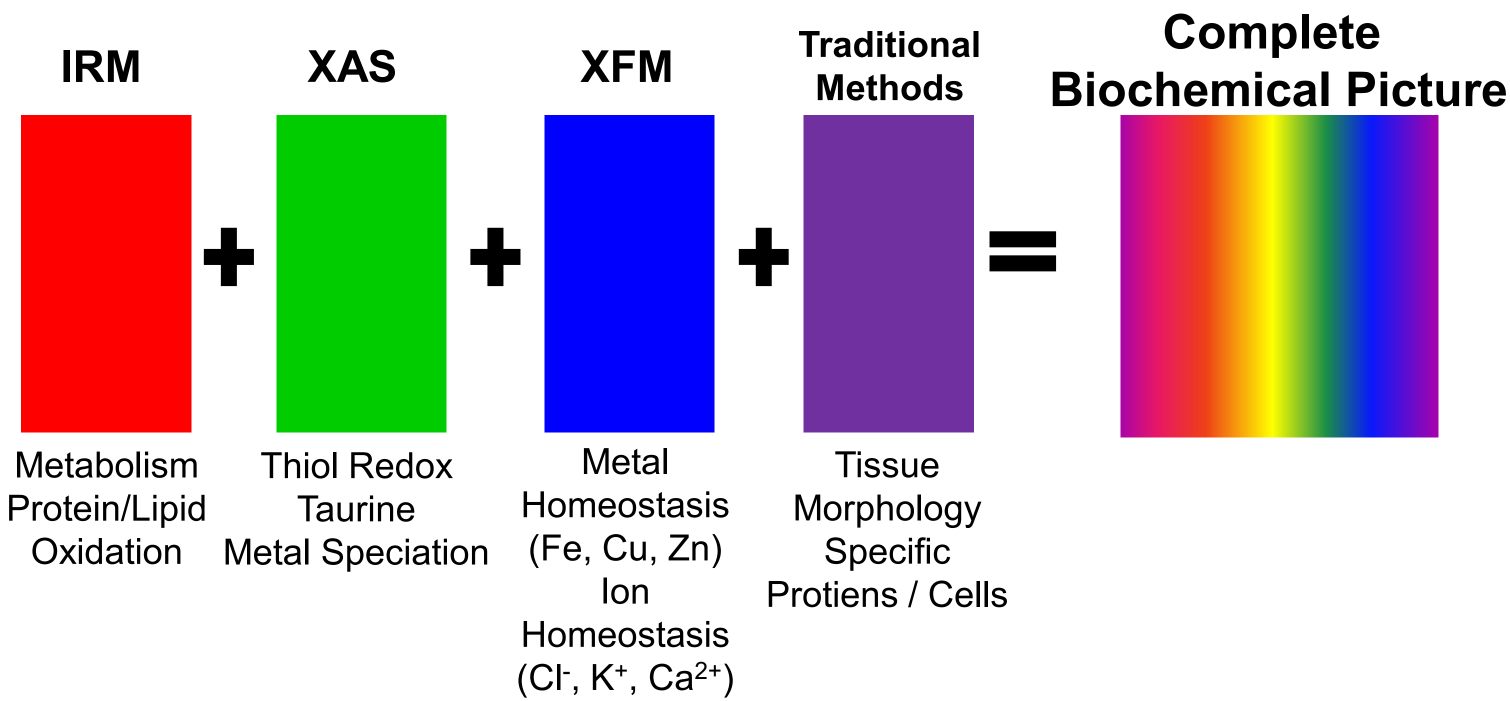
Assays
Bulk Techniques
Mass Spectrometry



High Spatial Resolution
Low Amount of Chemical Information

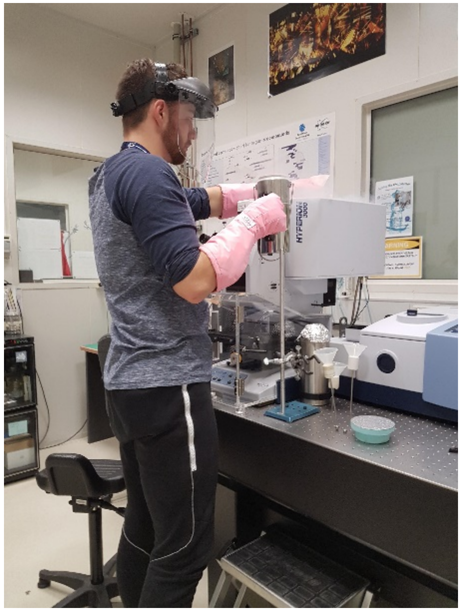
Poor Spatial Resolution
Information Rich

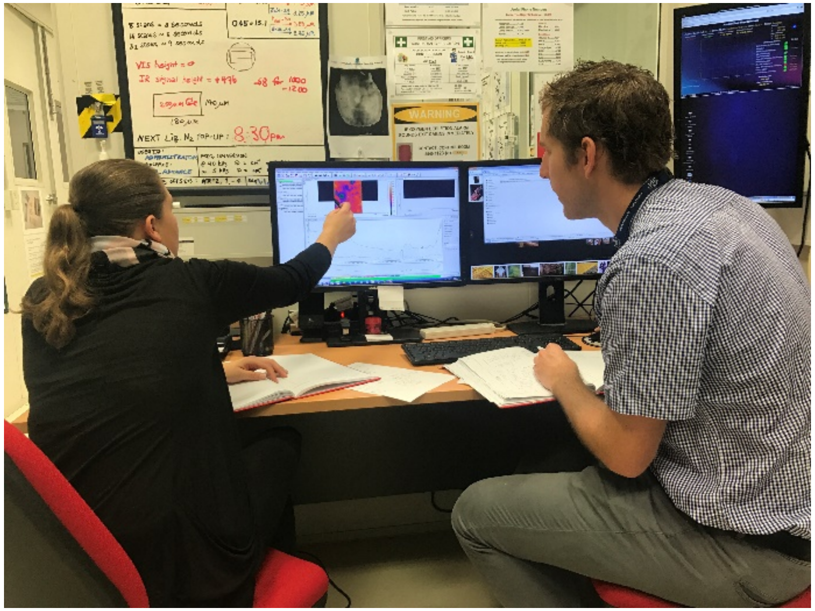
THE MULTI-MODAL APPROACH



WHY THE SYNCHROTRON??

A bright & tuneable light source!

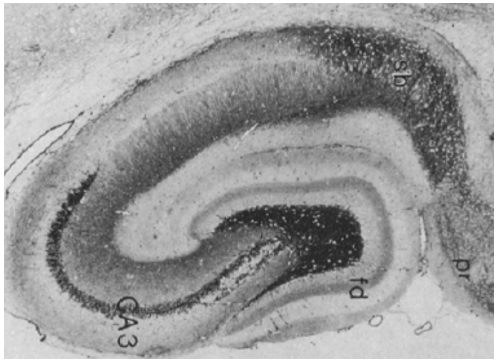




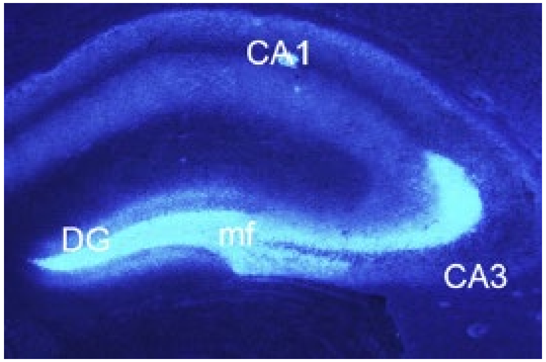
METAL HOMEOSTASIS AND BRAIN FUNCTION

- Zinc binding accelerates amyloid-β plaque formation
- Zn deficiency is associated with memory loss
- Brain Fe accumulates during ageing and neurodegenerative disease

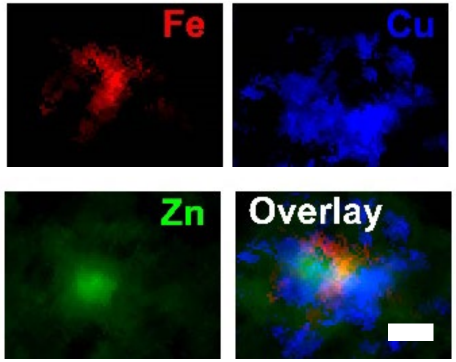
- **How do we study brain-metal homeostasis?**



Danscher et. al.,
Histochemistry 55, 27-40. **1978**



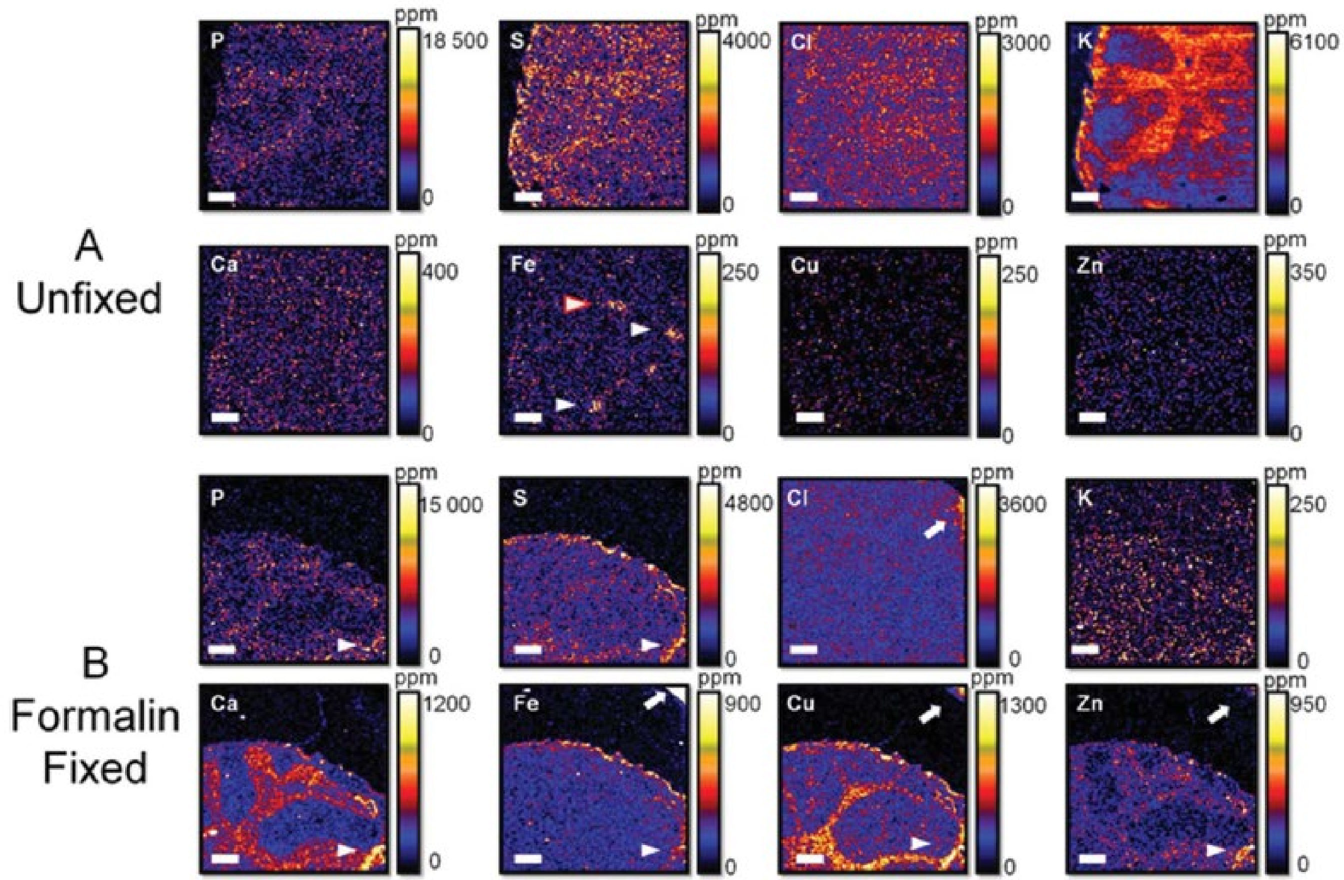
Lee, Joo-Yong, et. al.,
Brain research 1418. 12-22, **2011**



Summers et. al.,
Biochemistry, 56, 4107-4116 **2017**

EXPERIMENTAL CONSIDERATIONS FOR XFM/IRM REALLY IMPORTANT!

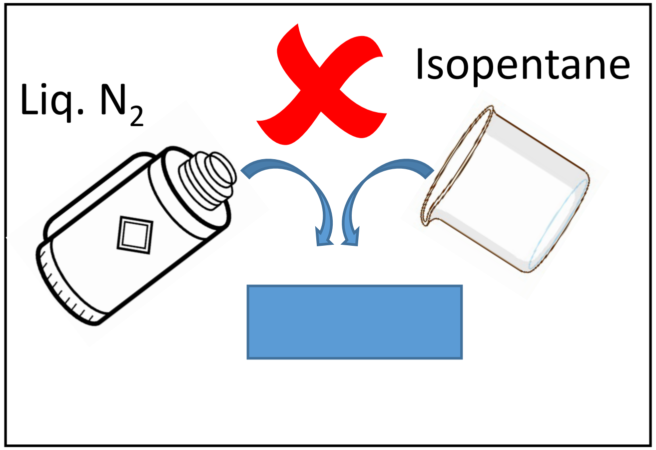
- Preparing tissues for XFM and IRM requires different preparation than histology and immuno-histochemistry

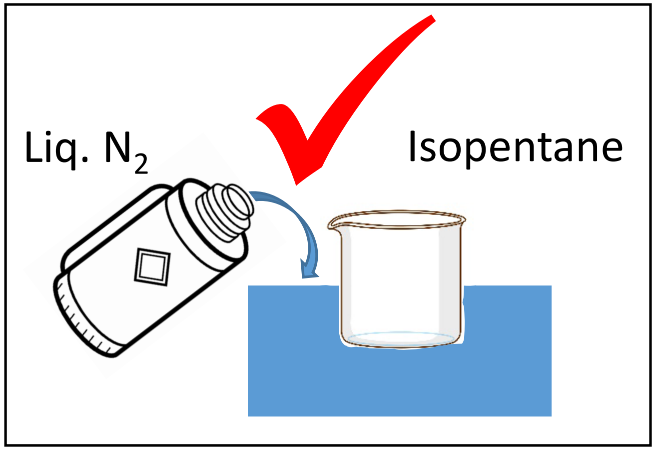


Hackett et. al., **Analyst**, 2011

SAMPLE PREPARATION – RAPID FLASH FREEZING

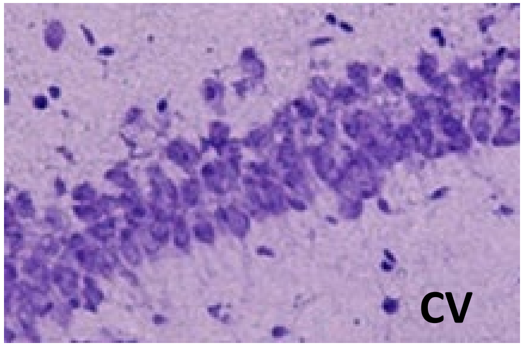
- Tissues samples are rapidly dissected and immediately flash frozen in liquid-nitrogen cooled iso-pentane
  - *(no perfusion, no fixation)*
- Samples stored in -80 ^{0}C until ~ 1 week before analysis
- Samples sectioned on a *cryo-mictrotome at -16 ^{0}C* for brain (liver -18 ^{0}C), *10-µm-thick*. This is warmer than most SOPs, which are written for sucrose-cryoprotected, formalin-fixed tissue
- Tissue sections “melted” onto substrate, and either *transferred back to -80 ^{0}C freezer, or air-dried*
- Analysis typically within 1 week of sectioning
- If your samples are cracking, or tearing when you section, they are too cold. It takes about 30 – 40 min for tissue to warm from -80 ^{0}C to – 16 ^{0}C in cryostat
- Substrates
  - 500 nm, 1 µm, or 2 µm thick Si_3N_4
    - *XFM, or multi-modal XFM/IRM*
  - 500 µm or 1 mm thick CaF_2, BaF_2
    - *For IRM*



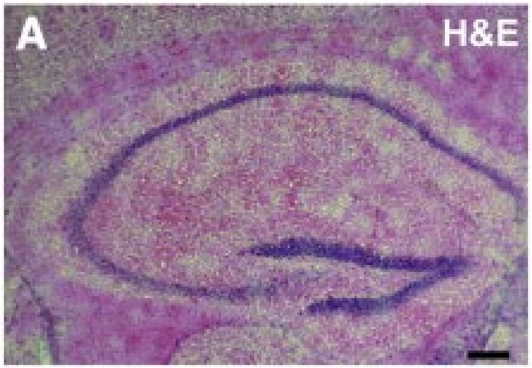


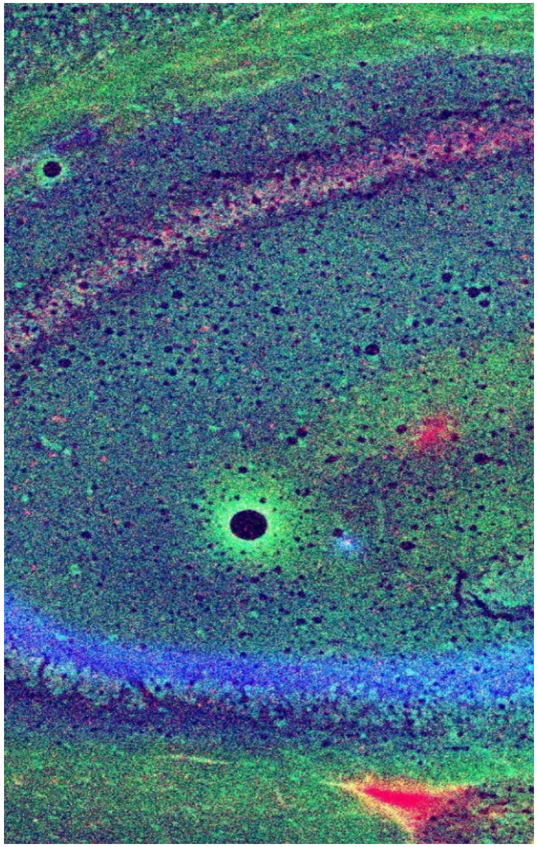
RAPID FLASH FREEZING, HISTOLOGY, & ICE CRYSTALS

- Histology and immuno-histochemistry can easily be performed on flash frozen tissues
- Either on the same tissue analysed at synchrotron, or serial sections
- Post-sectioning immersion fixation (typically 10 % PFA)

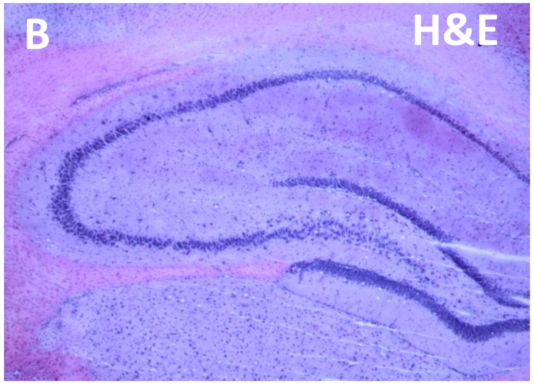


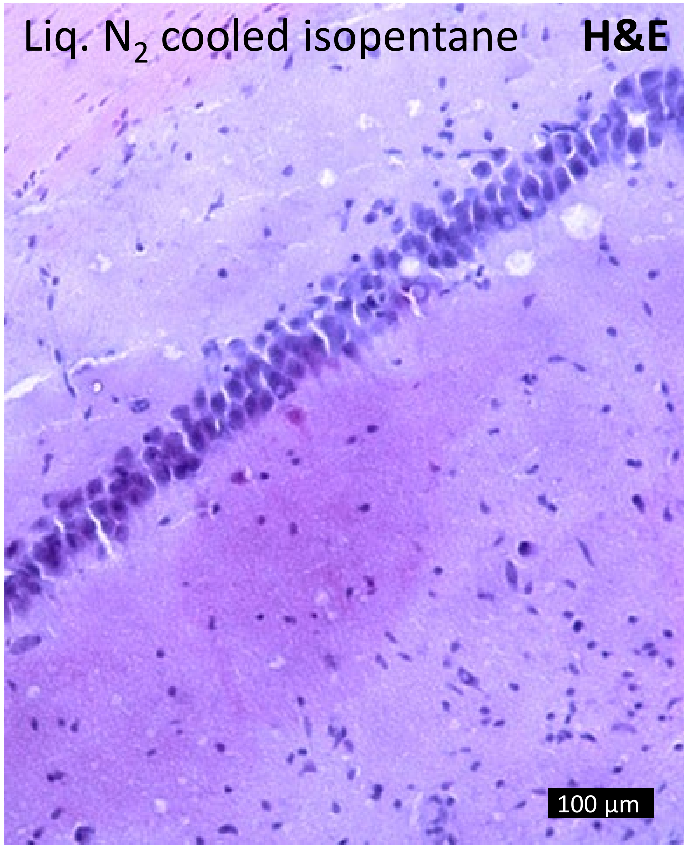
Freezing on dry ice





Liq. N_2 cooled isopentane

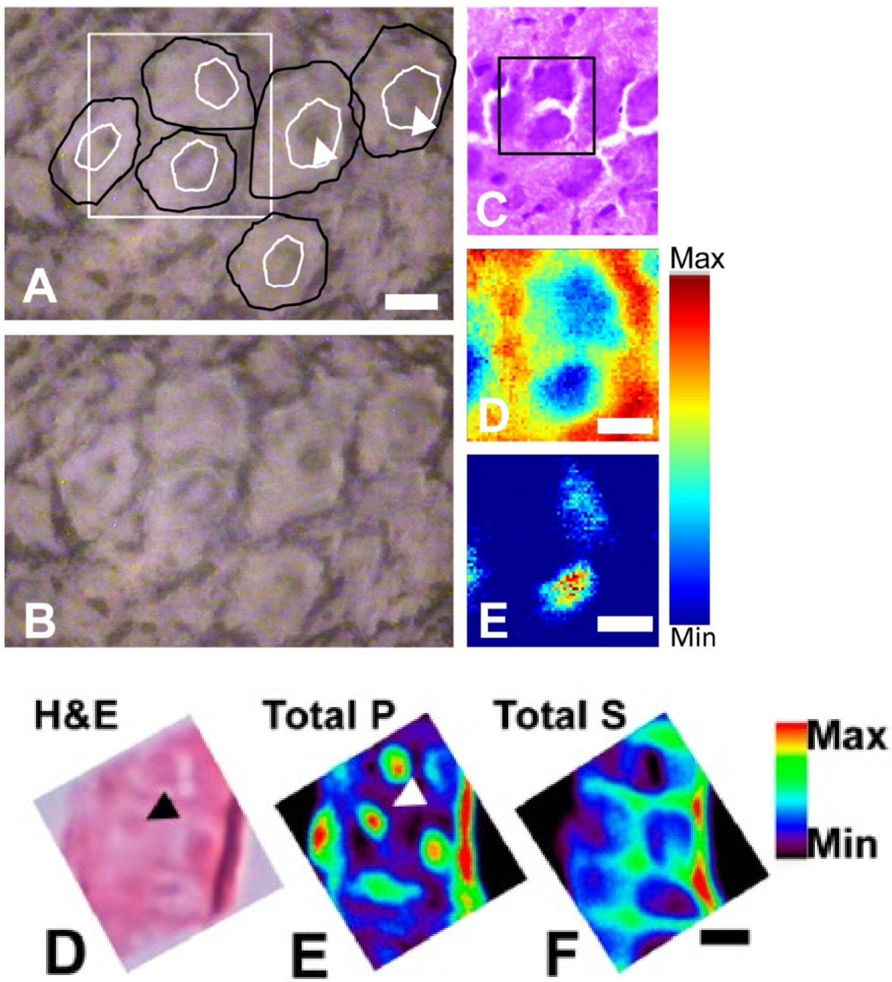




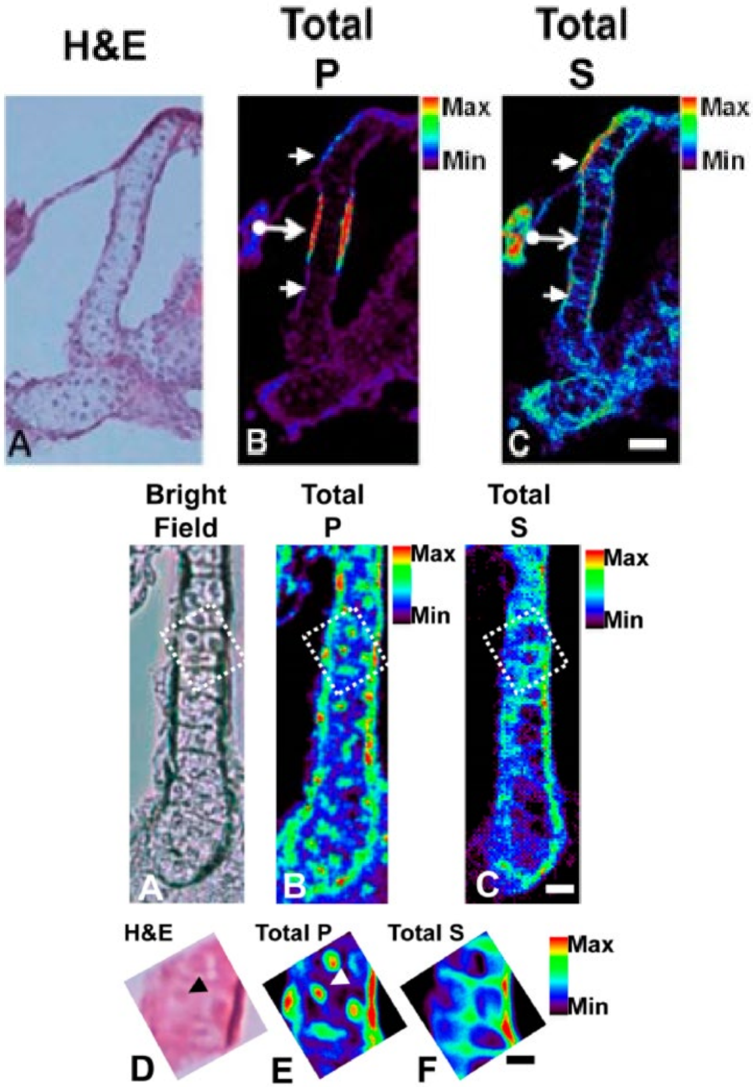
Neuroimage. 2011, 55(1): 32–38.

SYNCHROTRON IMAGING, HISTOLOGY (SAME SAMPLE)

Optical Microscopy on Unstained Tissue, IRM, and H&E staining (CaF_2)

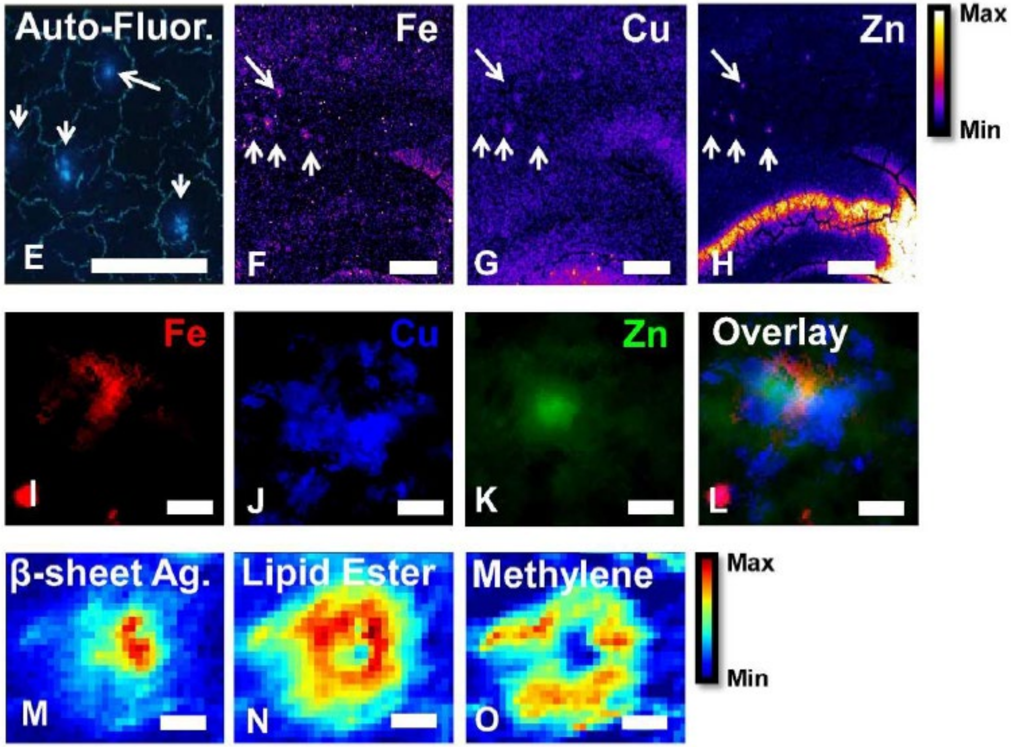


XFM (nano-probe) and H&E Histology (Si_3N_4)



MULTI-MODAL INVESTIGATION OF Aβ-PLAQUES

- Is there an association between Cu and lipids in plaque periphery?
- UV-autofluorescence, XFM (metal ions), IRM (protein aggregates, lipids)
- 10-µm-thick, air-dried tissue sections, Si_3N_4



Summers et. al., *Biochemistry*, 56, 4107-4116 **2017**

IMAGING BRAIN IRON HOMEOSTASIS DURNG AGEING

- Brain Fe increases (if you compare adult to senescent animals)
- Fe is redox active, does elevated brain Fe contribute to heightened oxidative stress?
- **Where is Fe accumulating in the brain, and in which cell types?**

WHY NOT JUST USE PERLS' IRON HISTOCHEMISTRY?

Reaction of Ferric Iron with Ferrocyanide

Perls Stain: $4Fe^{3+} + 3[Fe(CN)_6]^{4-} \longrightarrow Fe^{III}[Fe^{III}Fe^{II}(CN)_6]_3$

Requires dilute acid

Does not stain all forms of iron (no heme, no labile Fe)

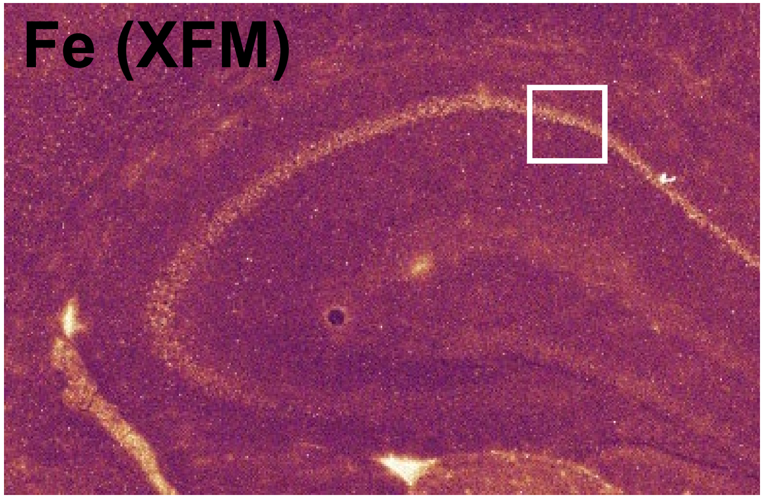
Not quantitative

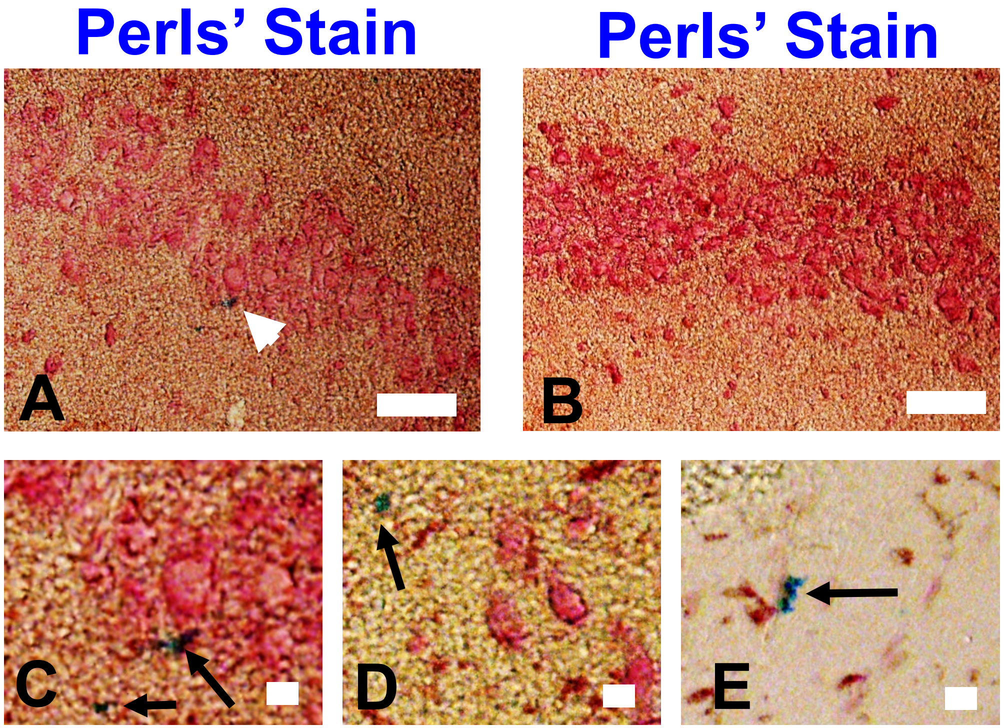
Ferrihydrite

$(Fe^{3+})_2O_3.5H_2O$

ARE NEURONS ENRICHED IN IRON? (YES)

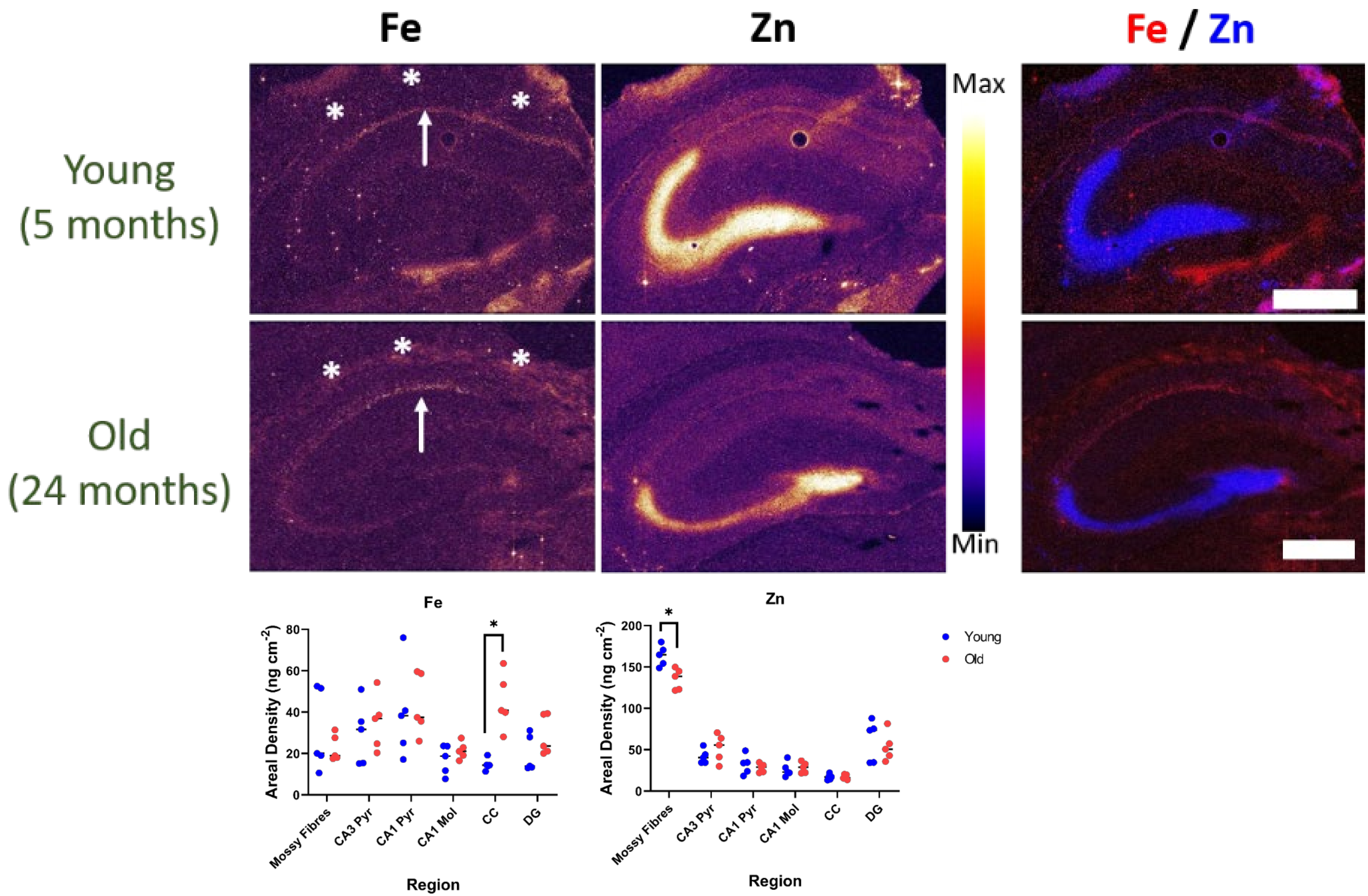
Conflicting results between elemental mapping and Perl's histochemistry?





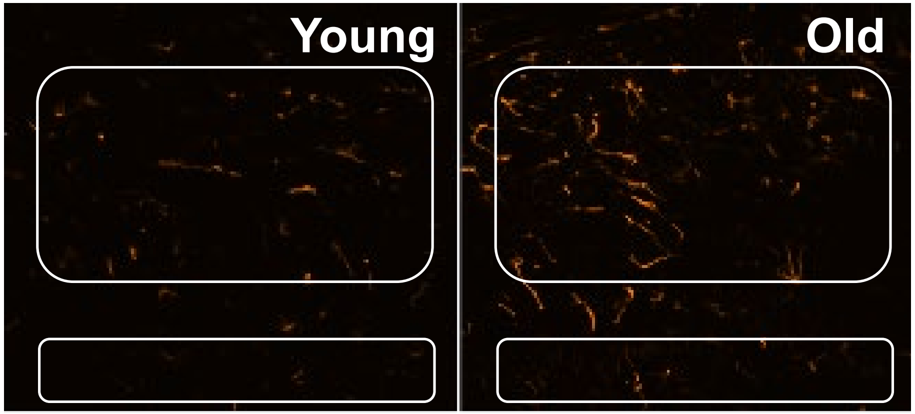
Hackett et. al. *Metallomics 11, 151-65, **2019***

BRAIN IRON INCREASES IN WHITE MATTER DURING NATURAL AGEING

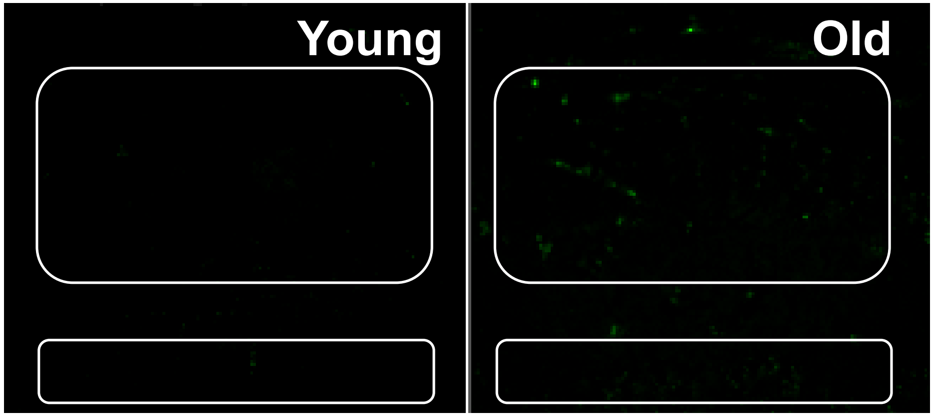


COMBINING XFM WITH IMMUNO-FLUORESCENCE

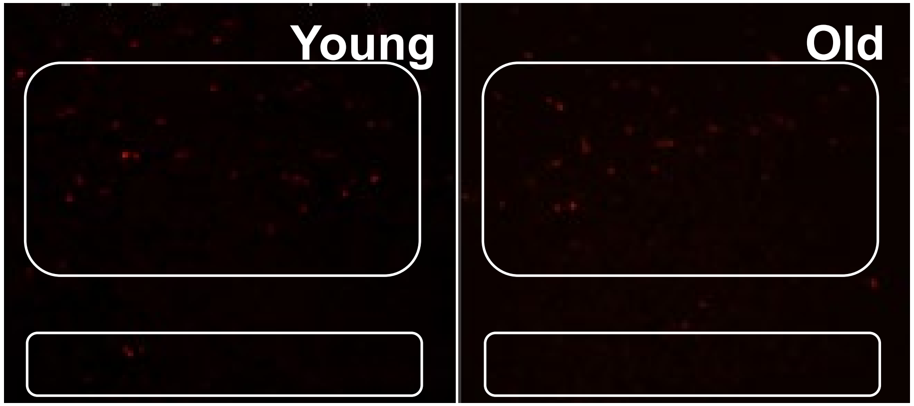
GFAP (astrocytes)



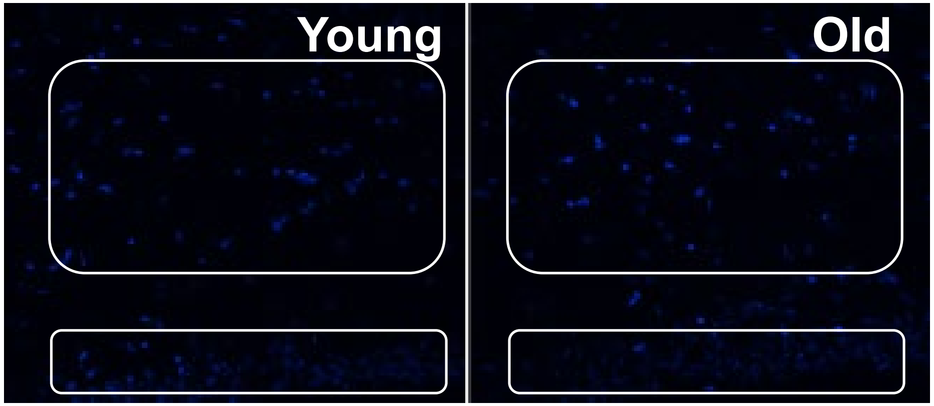
IBA1 (microglia)



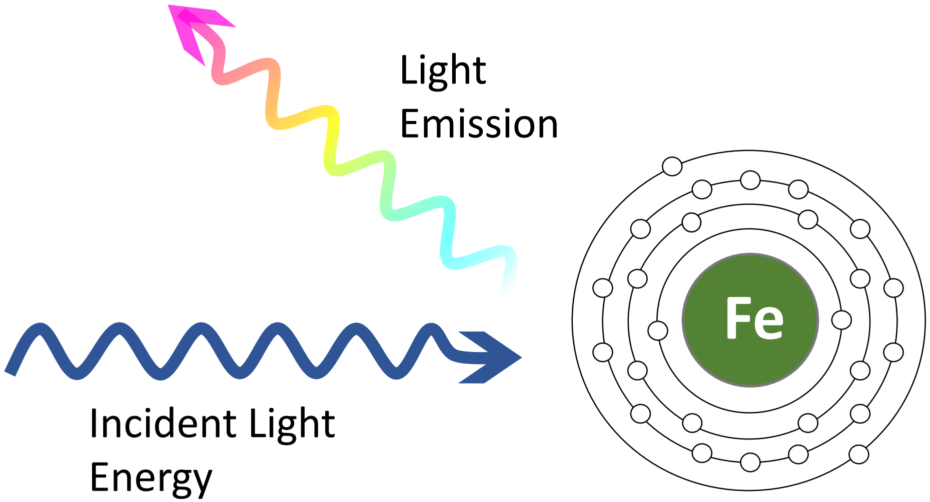
Olig2 (oligodendrocytes)



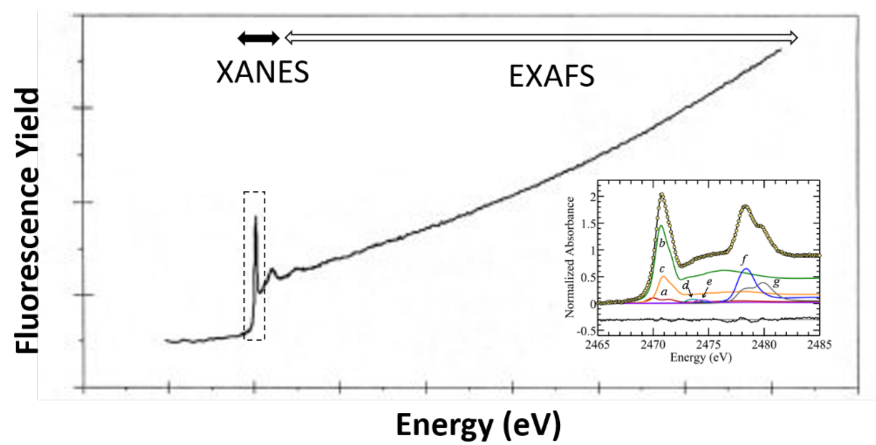
DAPI (nuclei)



CHEMICALLY SPECIFIC XANES-IMAGING



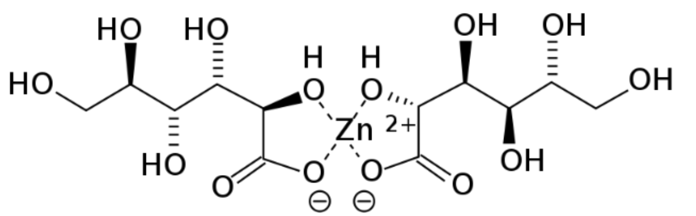
EXAFS: Theory is well understood, **Slow!**
XANES: Theory is not as well understood **Fast!**

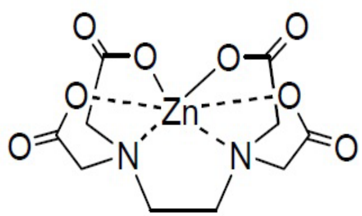


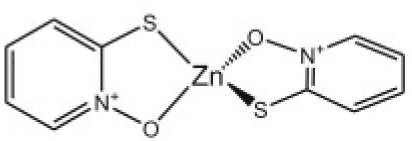
Animation courtesy of Nicholas Fimognari

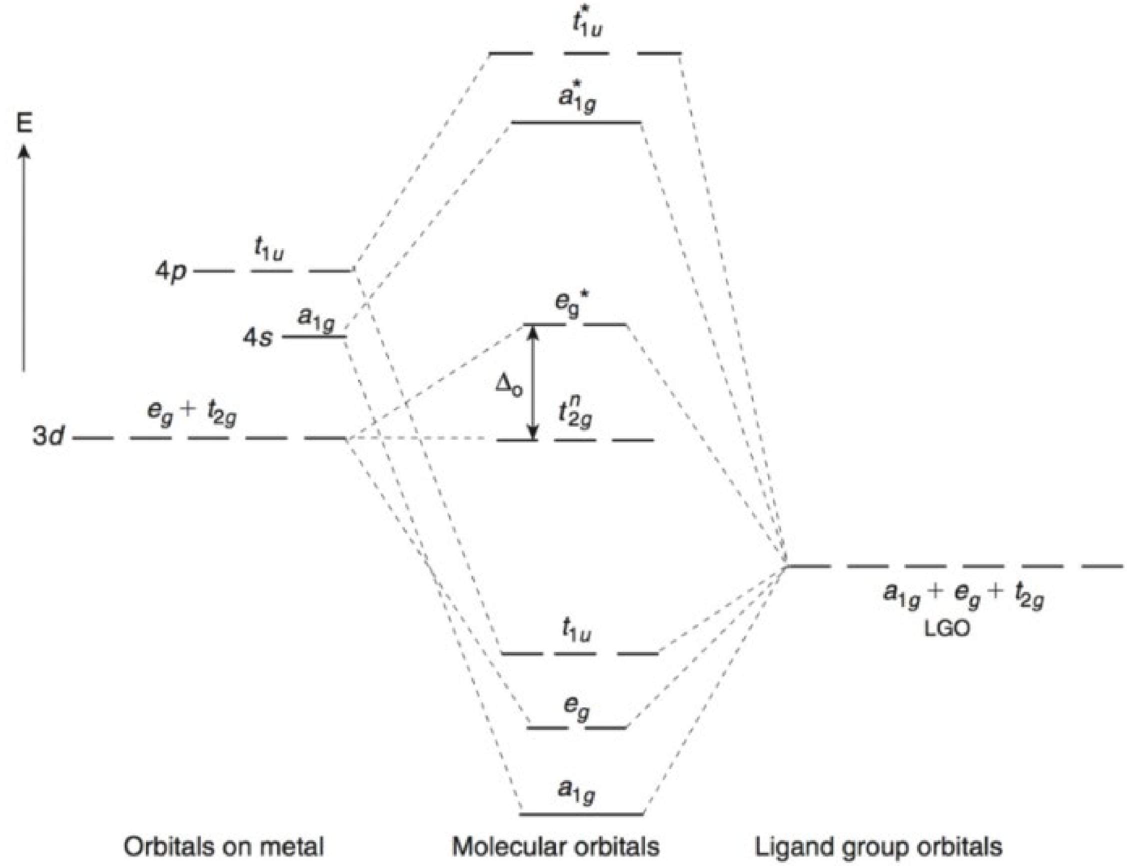
DEVELOPMING ZINC XANES IMAGING

XANES spectroscopy should be highly sensitive to the coordination geometry, number of ligands, and ligand type





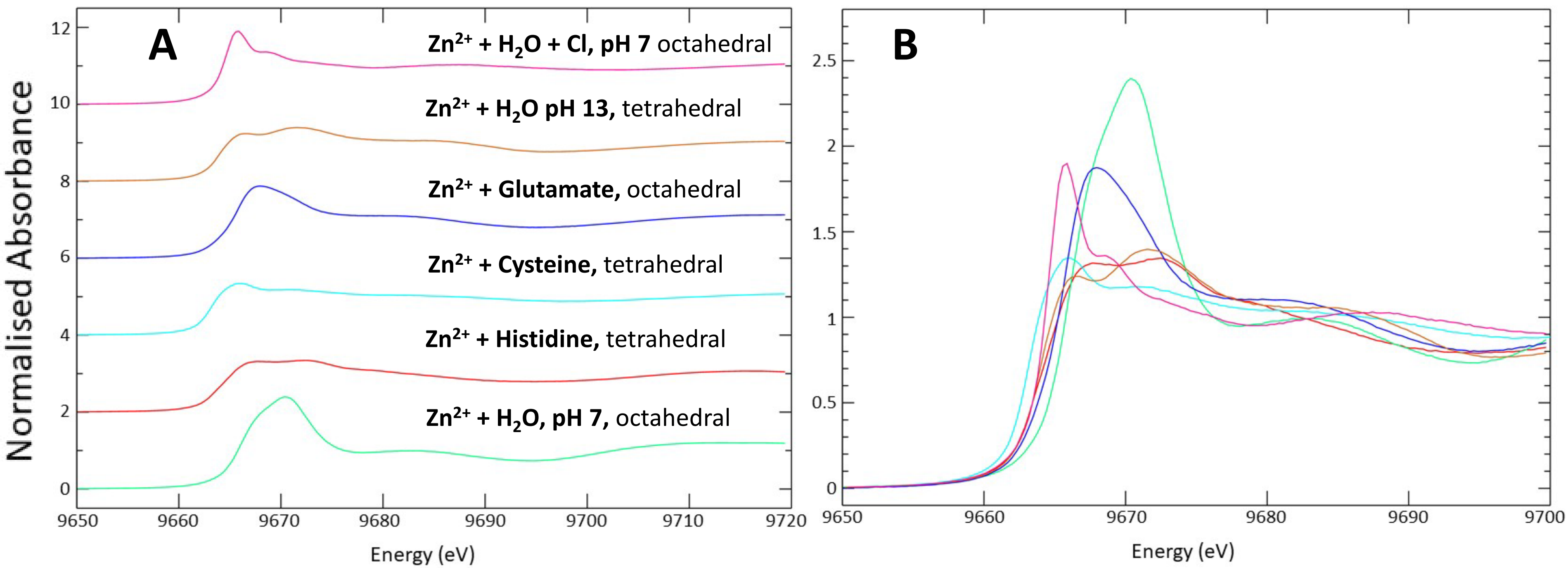




Archives of Biochemistry and Biophysics 611 (**2016**) 3-19.

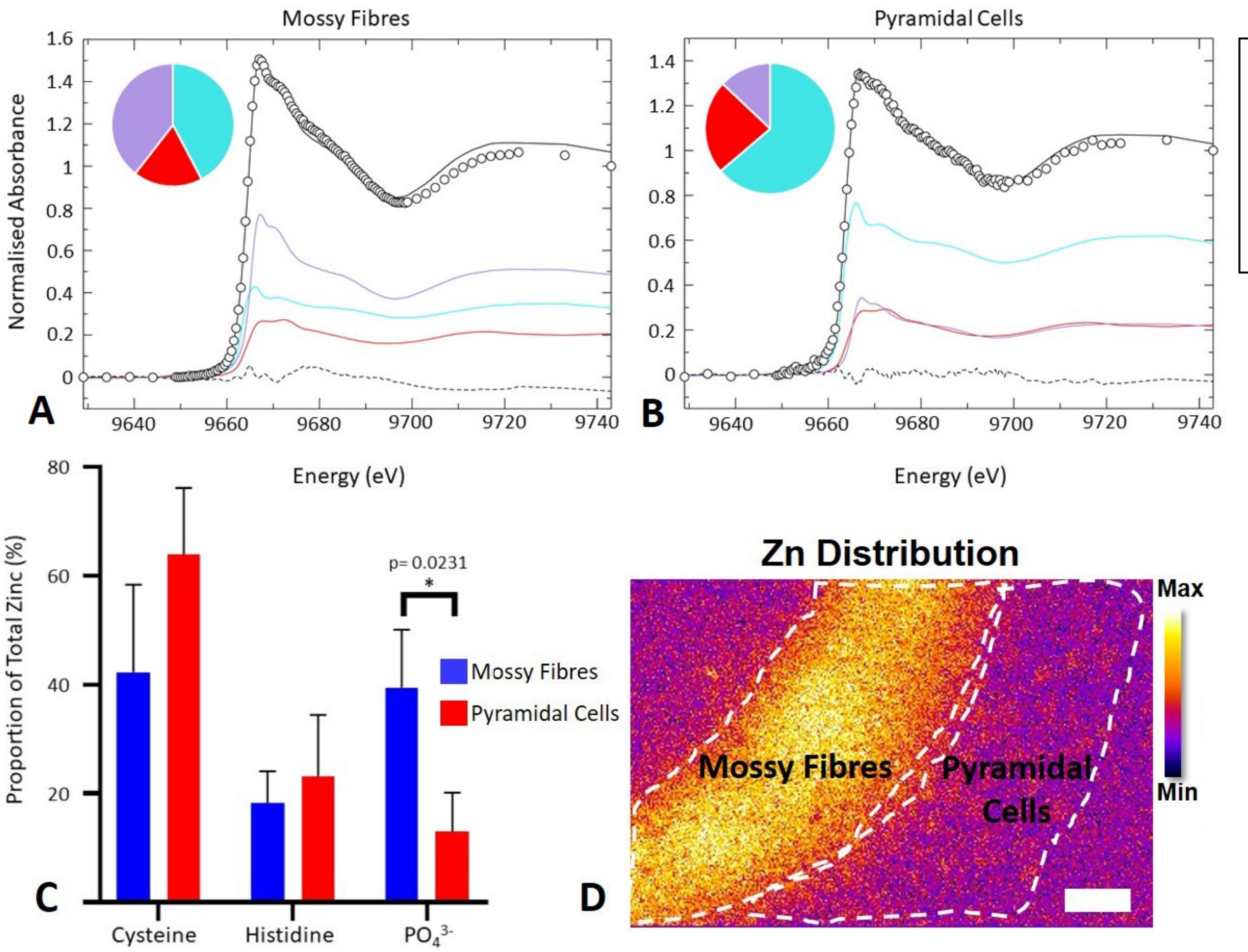
IS THE ZINC K-EDGE SENSITIVE TO COORDINATION CHEMISTRY? (YES)

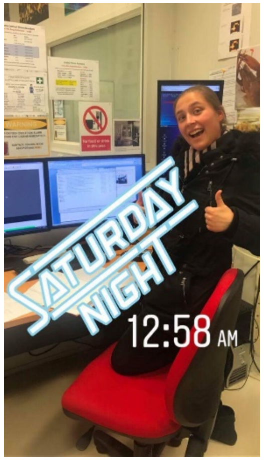
Modelling Zn Bonding in Biological Environments:
Zn^{2+} in presence of excess biological ligands



Hollings et. al., *Metallomics*, 12, 2143-2144, **2020**.

ZINC SPECIATION IN BRAIN TISSUE

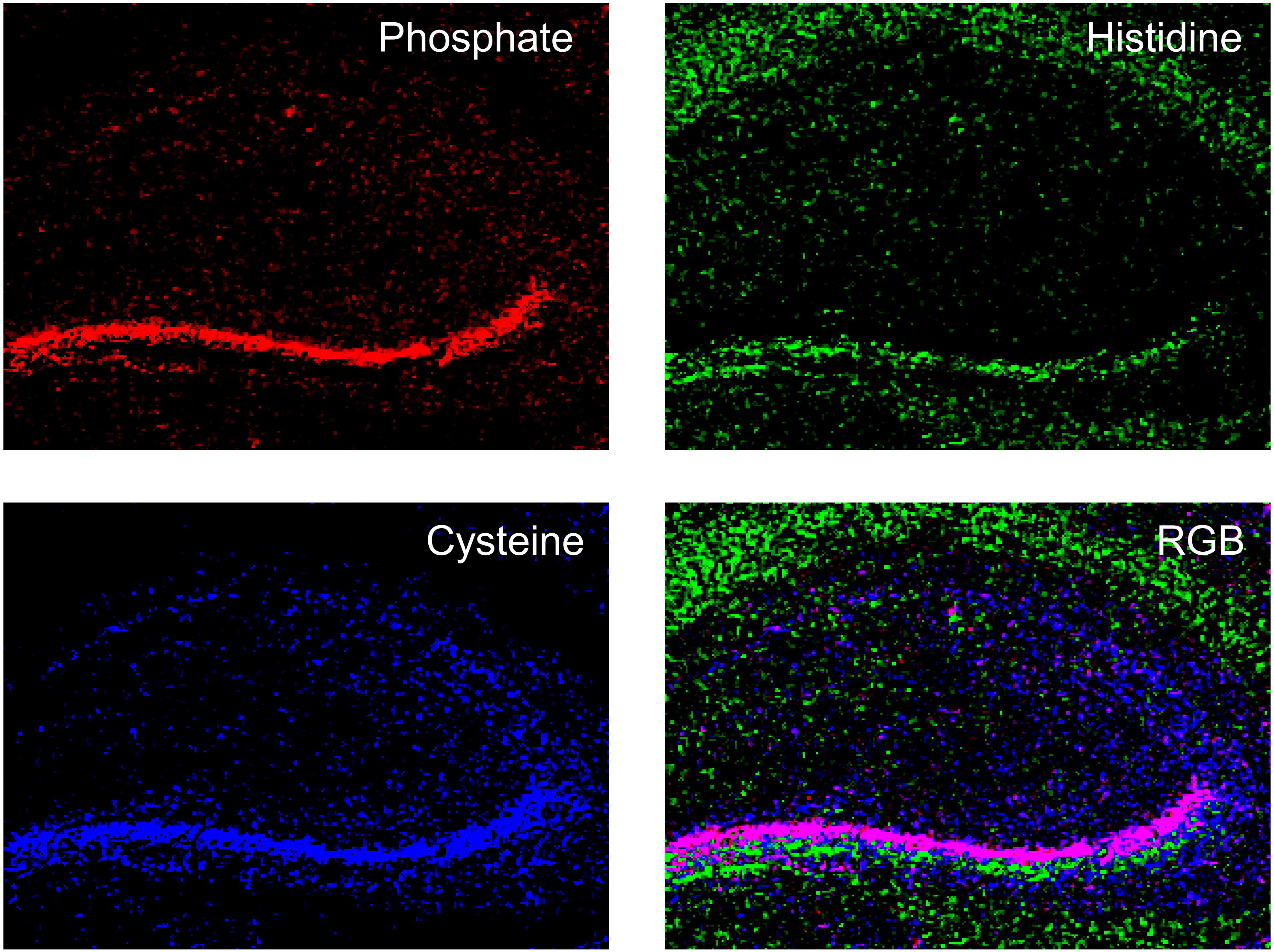




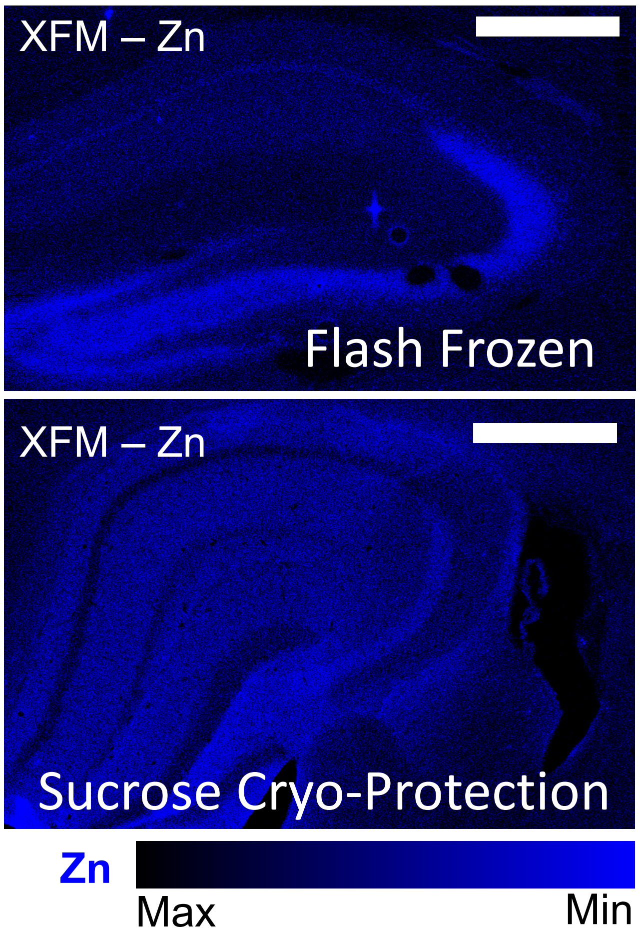
Hollings et. al., *Metallomics*, 12, 2143-2144, **2020**.

Ashley Hollings PhD Student

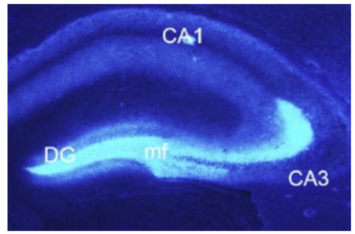
CHEMICALLY SPECIFIC XANES ZINC IMAGING



A CAUTIONARY TALE: SAMPLE PREPARATION & ANALYSIS CONDITIONS ARE CRITICAL!!

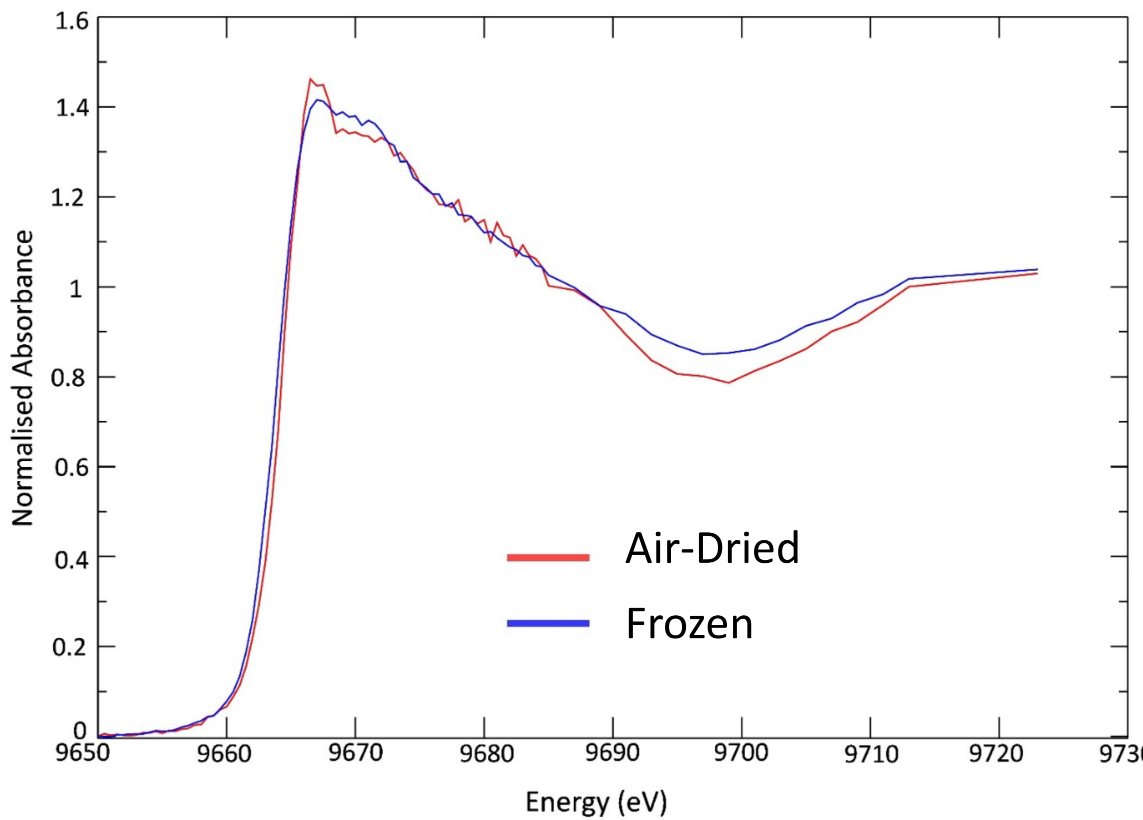


Scale = 500 µm



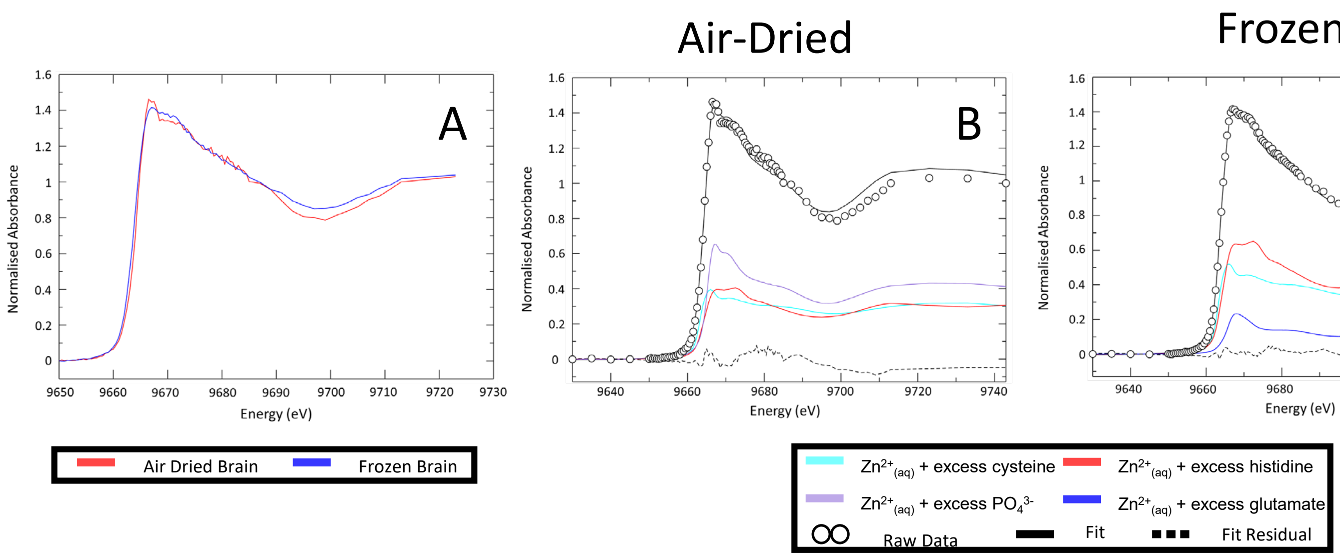
Lee, Joo-Yong, et. al., *Brain research* 1418, 12-22, **2011**

Hollings et. al., *JAAS*, 35, 2498-2508. **2020**

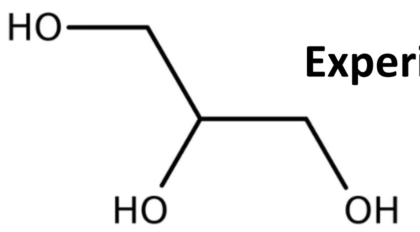


EFFECT OF AIR-DRYING

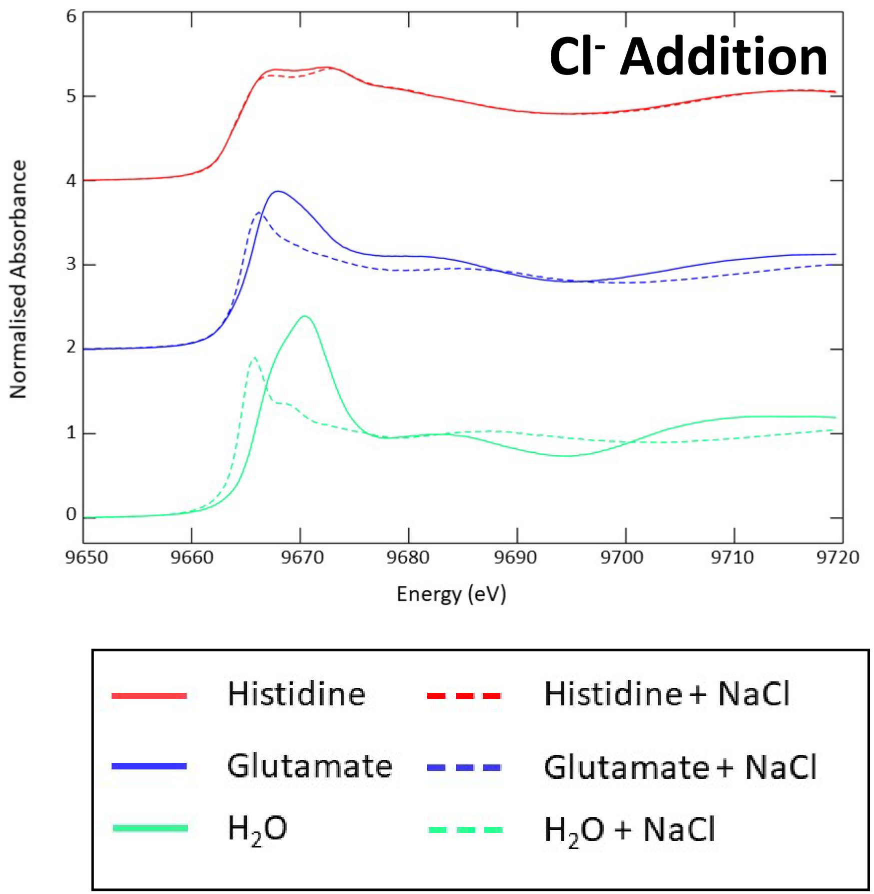
Air-drying tissue sections changes Zn^{2+} speciation, favouring coordination of Zn^{2+} with phosphates (not observed in frozen tissues)

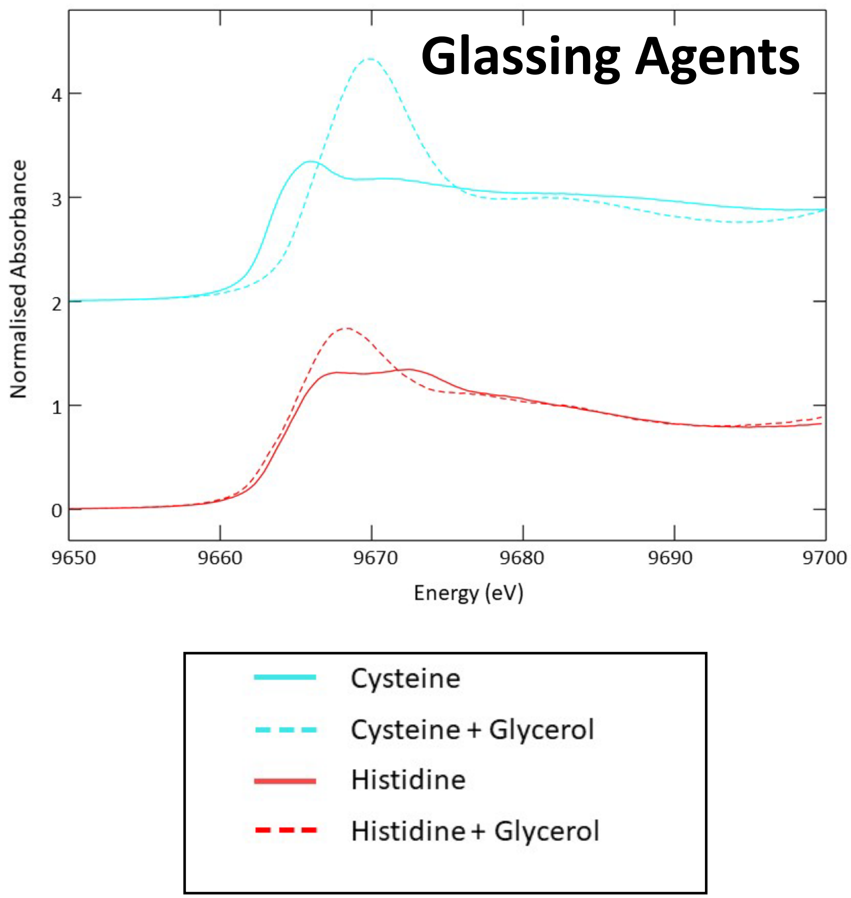


A CAUTIONARY TALE: SAMPLE PREPARATION & ANALYSIS CONDITIONS ARE CRITICAL!!

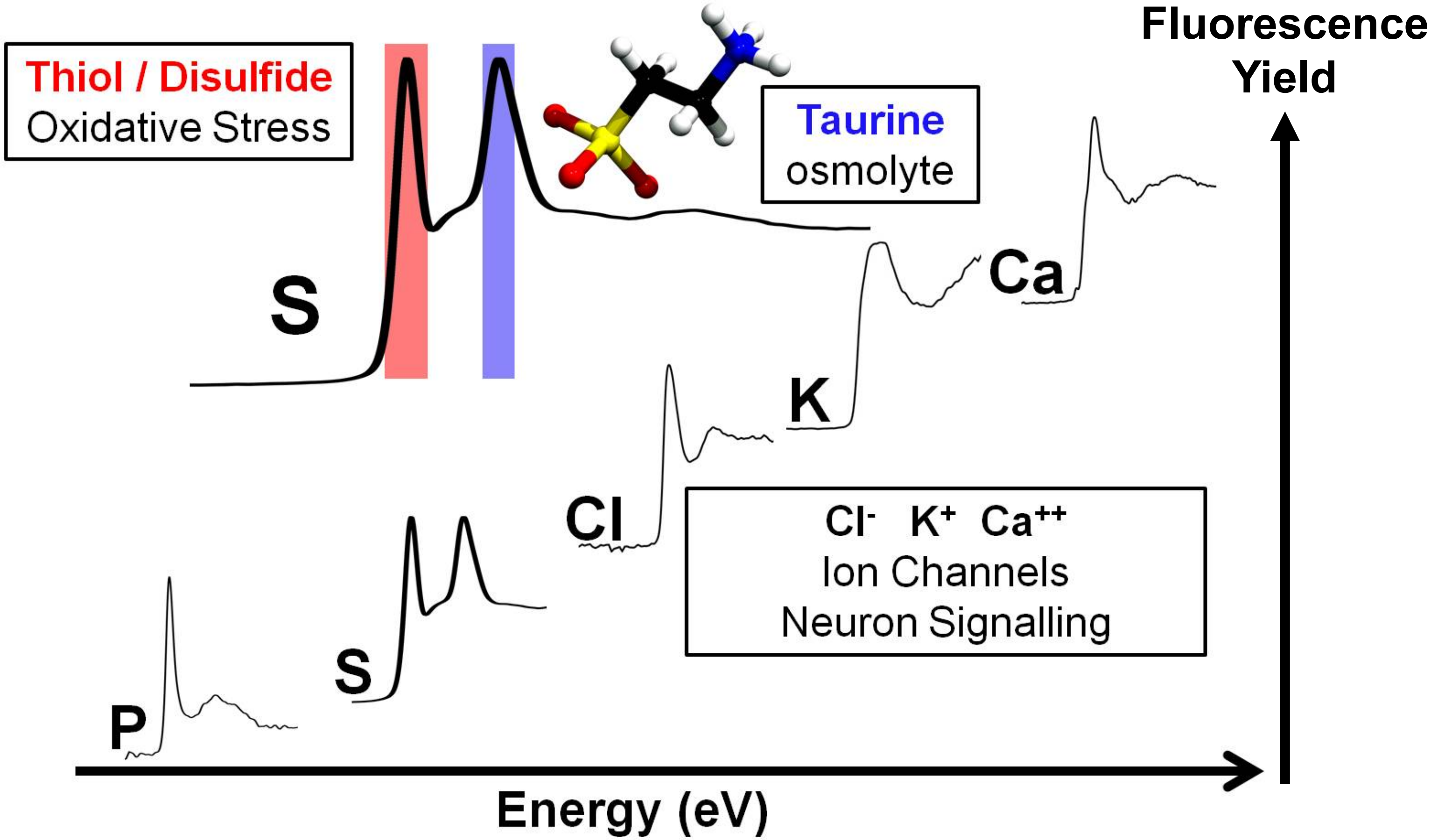


Experimental considerations for the XANES library: buffer ions, and "glassing agents"



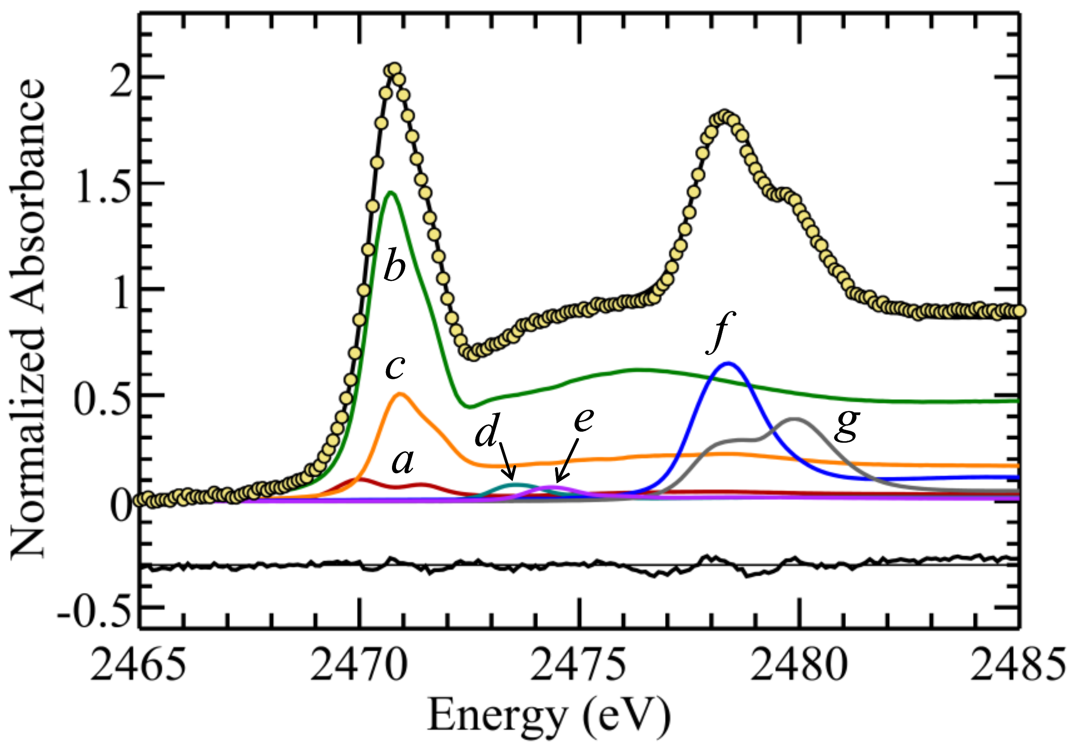


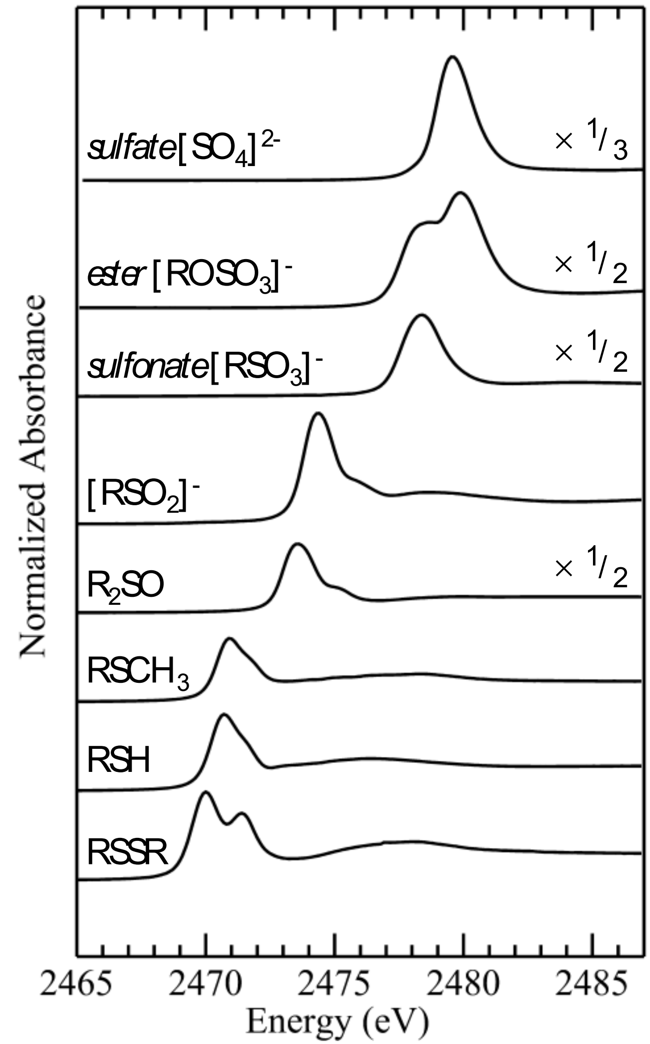
MEDIUM ENERGY X-RAY ABSORPTION SPECTROSCOPY (MEX) @ THE SYNCHROTRON



MEDIUM ENERGY X-RAY ABSORPTION SPECTROSCOPY

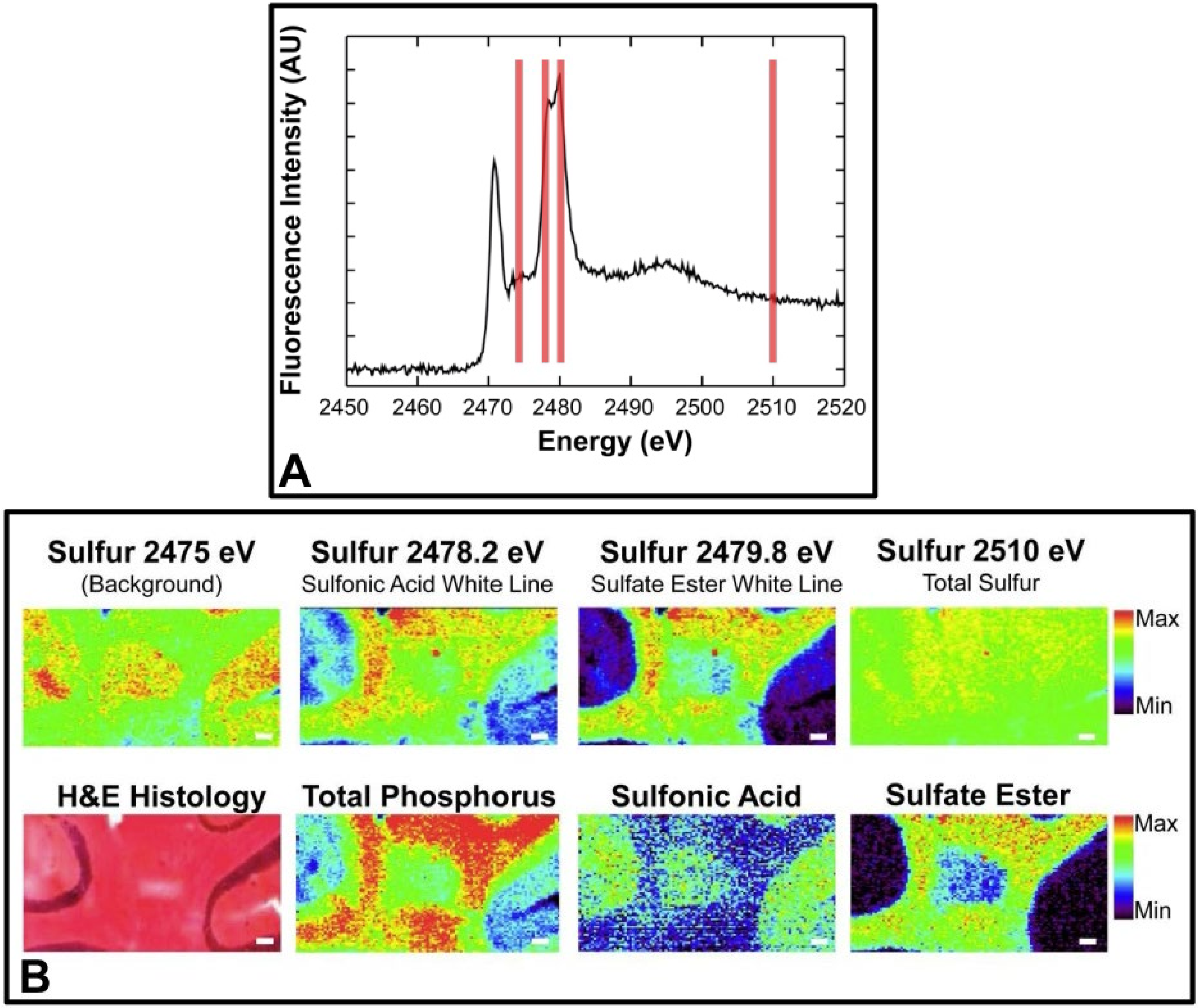
MEX Beamline: BRIGHT





Hackett et. al., *ACS Chemical Neuroscience*, 3 178-185, **2012**

MEDIUM ENERGY X-RAY ABSORPTION SPECTROSCOPY

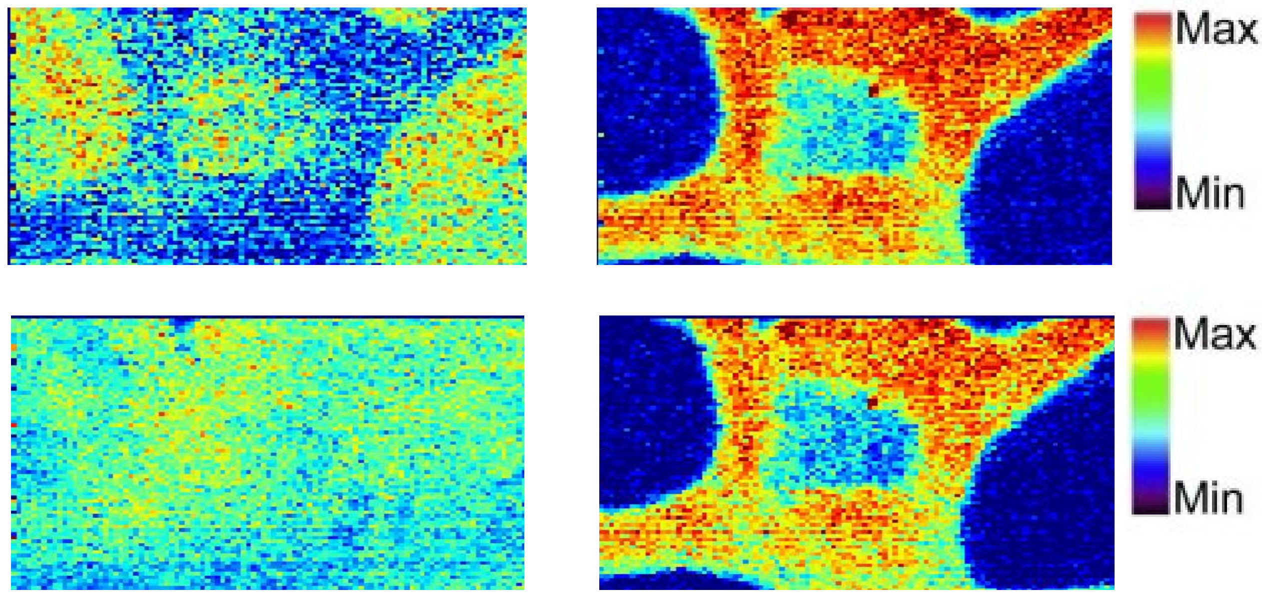


Pit falls with chemically specific XFM

Sulfonic Acid | **Sulfate Ester**

Good Energy Calibration (within 0.1 eV)

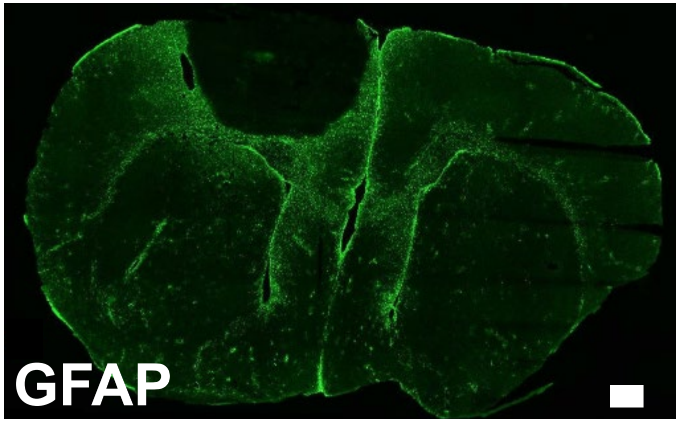
Bad Energy Calibration (off by 0.5 eV)



Energy Calibration & Stability is Critical!

Hackett et. al. (2016) Analytical Chemistry
Hackett et. al. (2016) Biochemistry

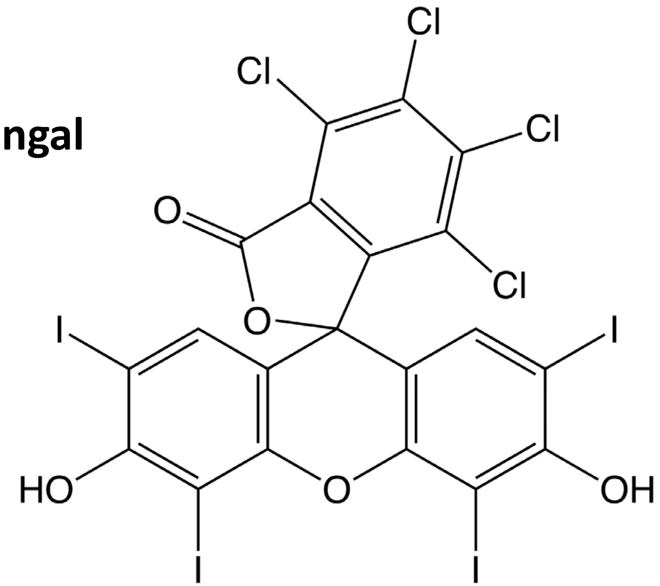
MEX: IS THERE AN ISCHEMIC PENUMBRA IN THE MURINE PHOTOTHROMBOTIC STROKE MODEL?



Scale = 500 µm

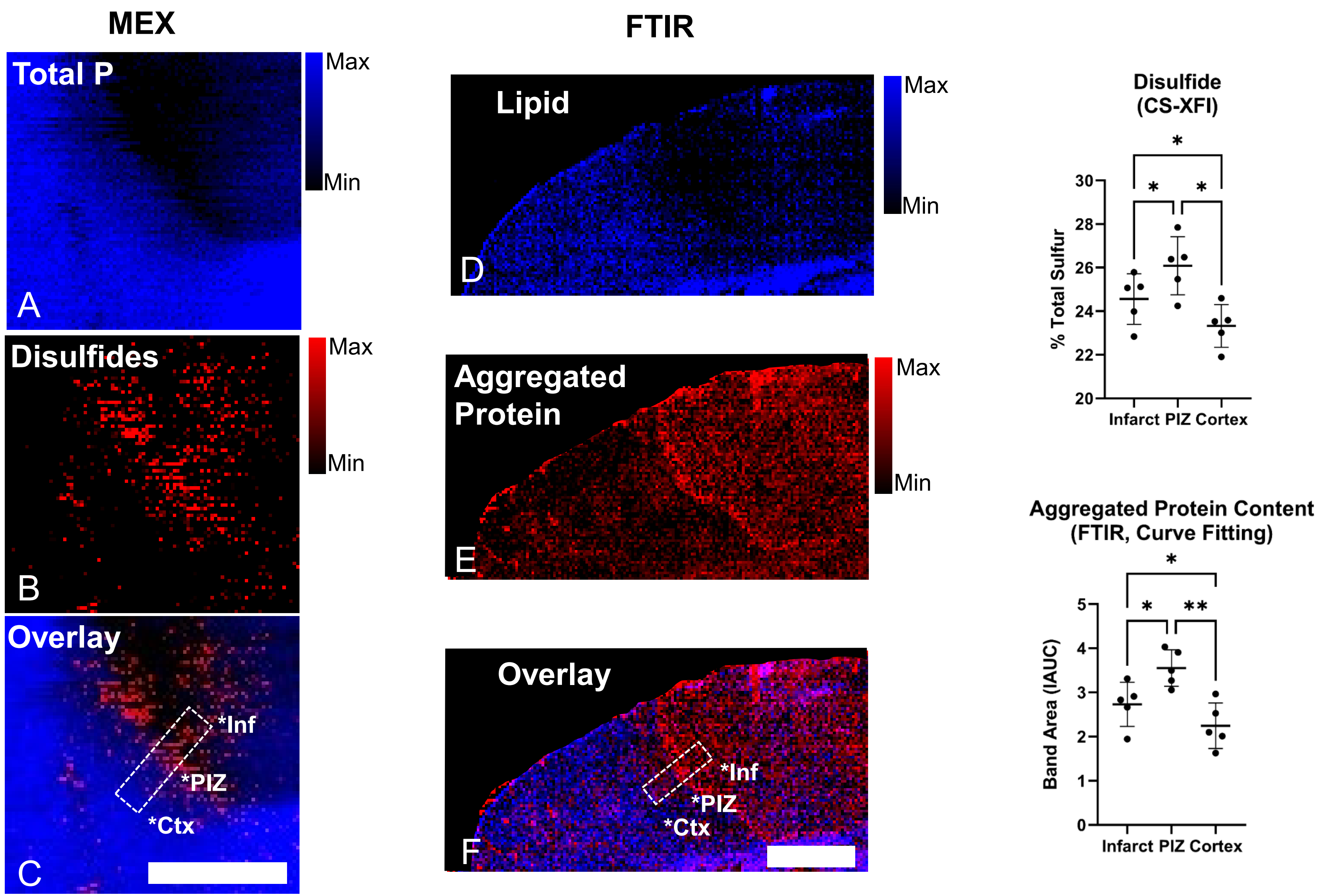
Saskatchewan Cerebrovascular Unit

Rose Bengal

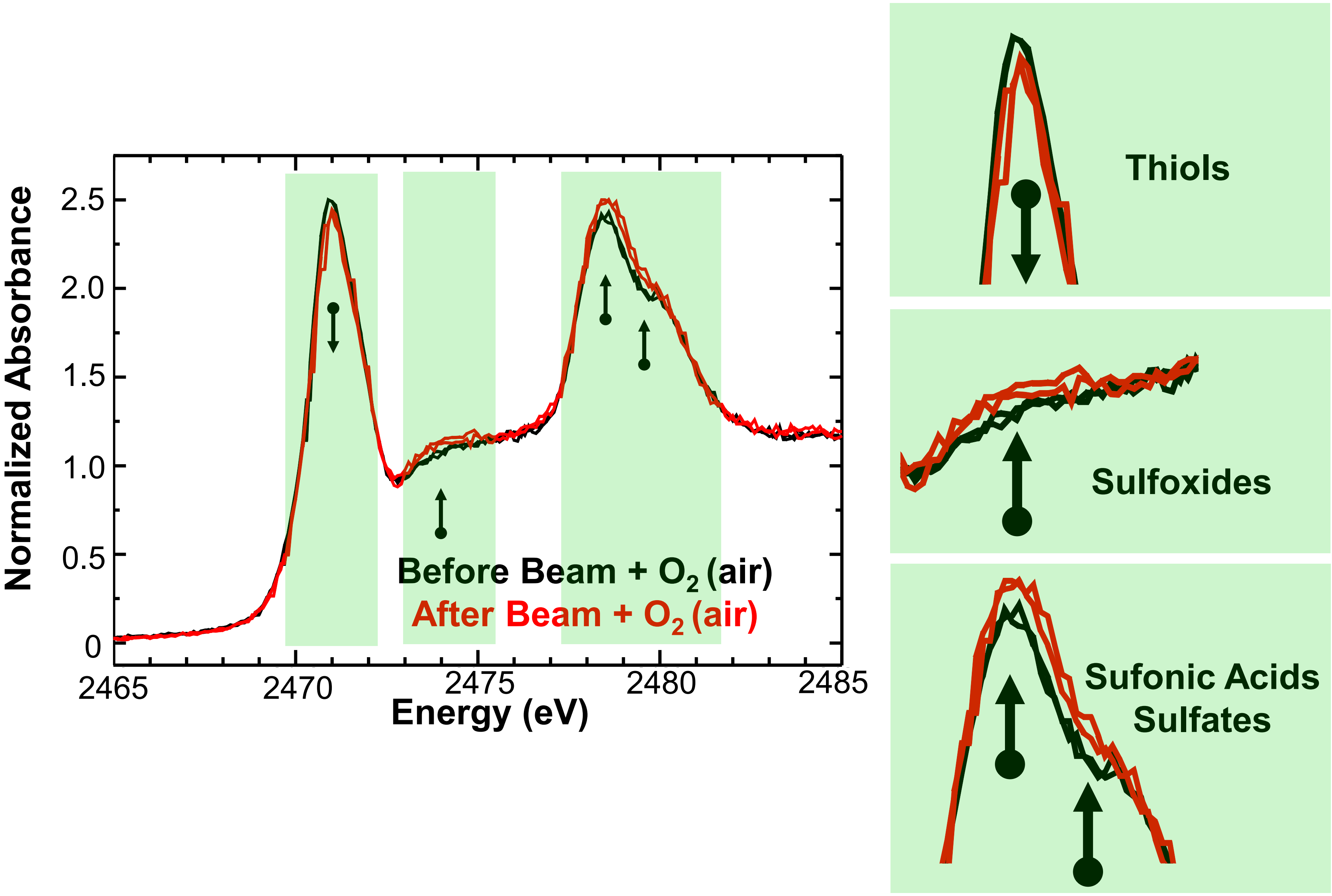


Is there an ischemic penumbra in the phototthrombotic stroke model?

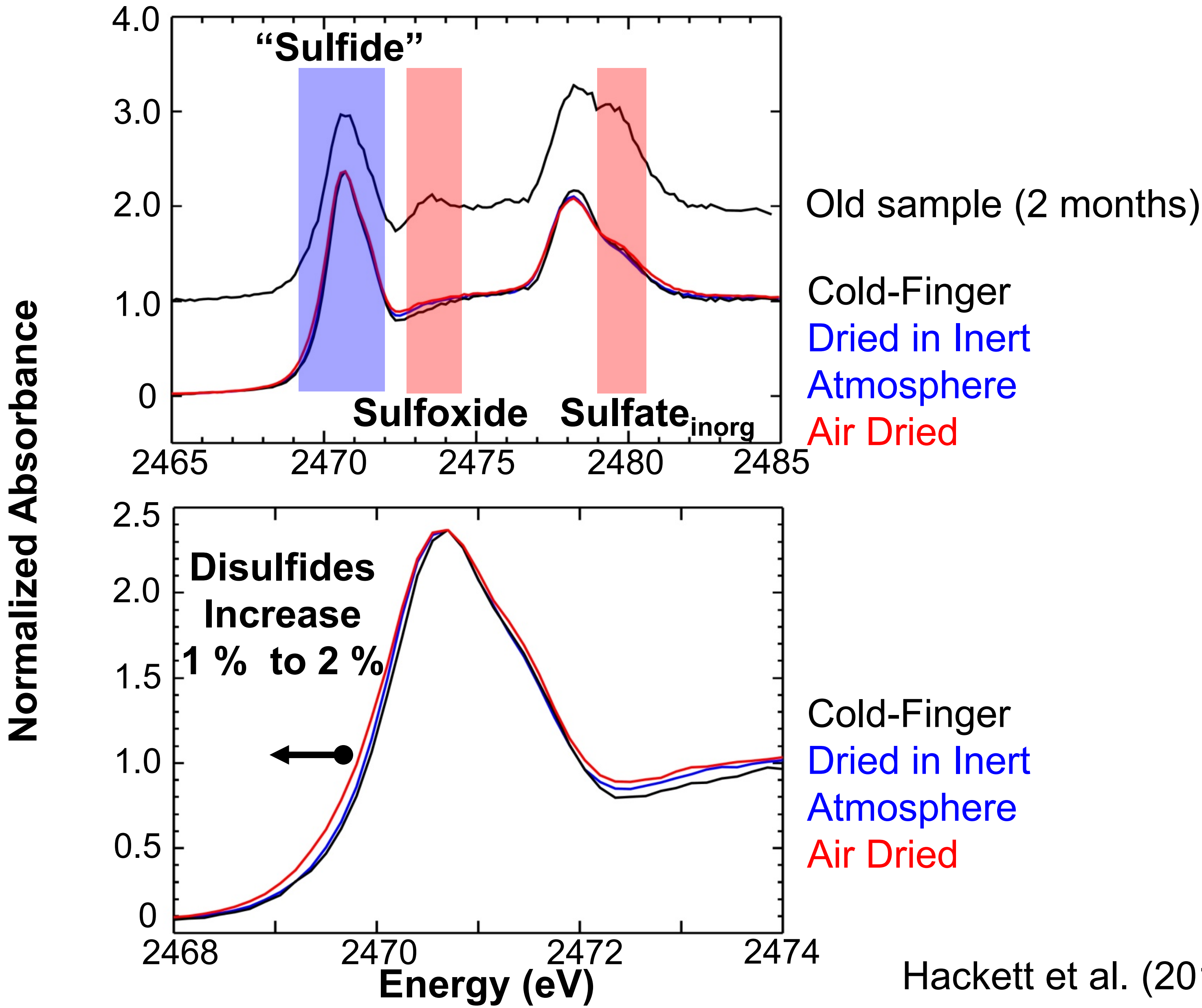
MEX: IS THERE AN ISCHEMIC PENUMBRA IN THE MURINE PHOTOTHROMBOTIC STROKE MODEL?



X-RAY PHOTO-DAMAGE



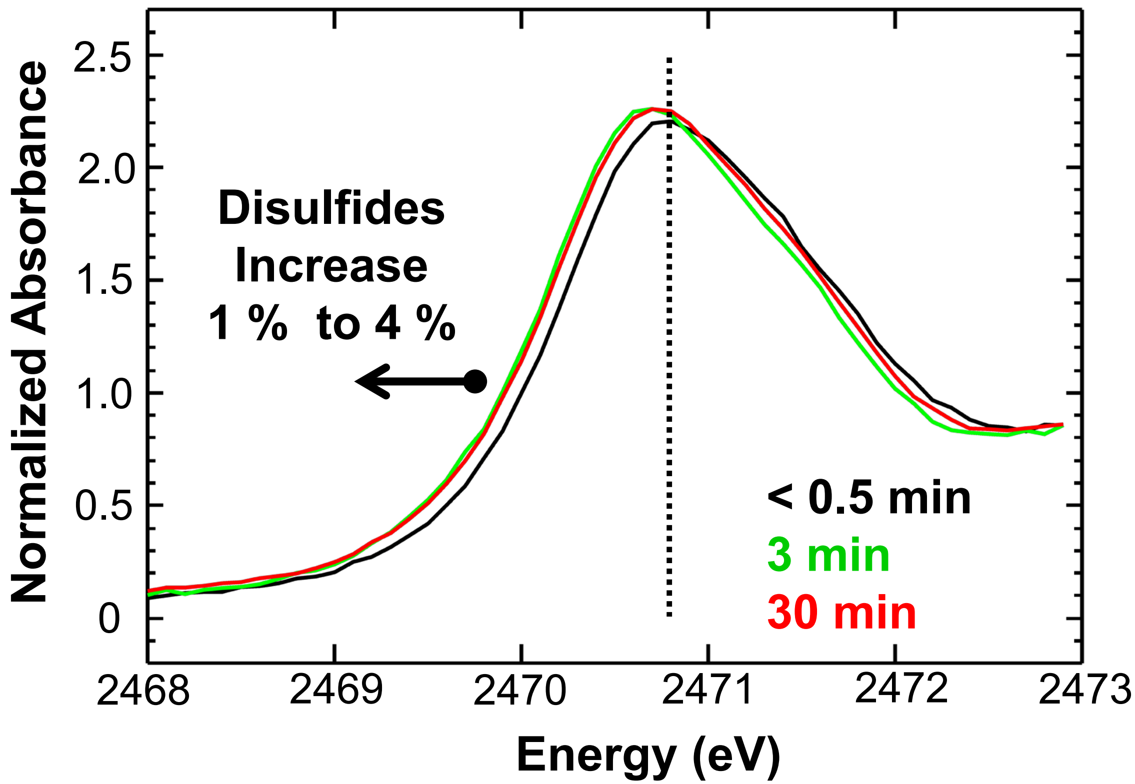
Effects of sample preparation



Hackett et al. (2017) J. Syn. Radiat.

EFFECTS OF POST-MORTEM INTERVAL

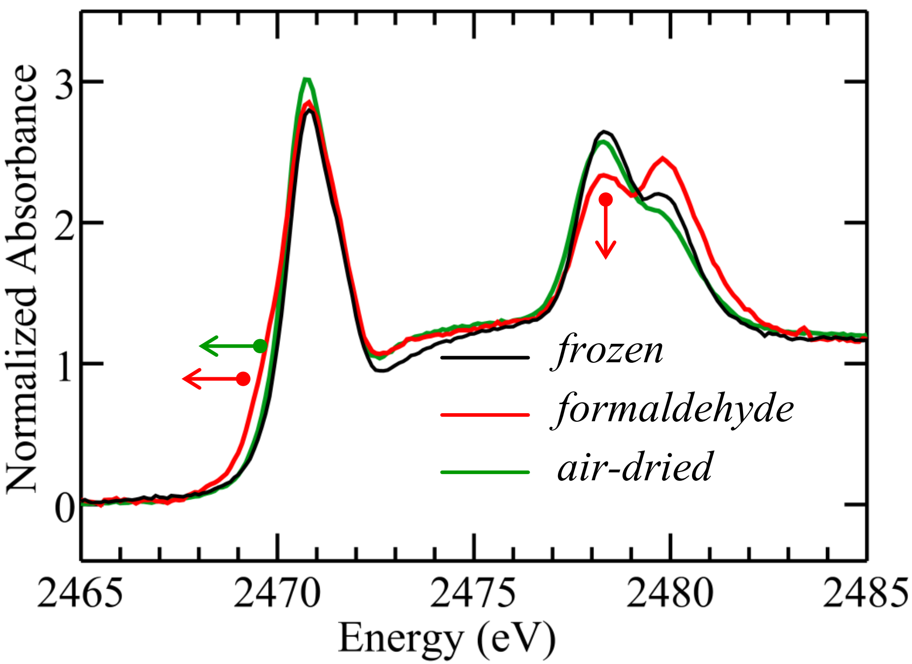
The time it takes to sacrifice an animal and remove its brain (30 seconds – 3 minutes) induces ischemia and oxidises thiols to disulfides

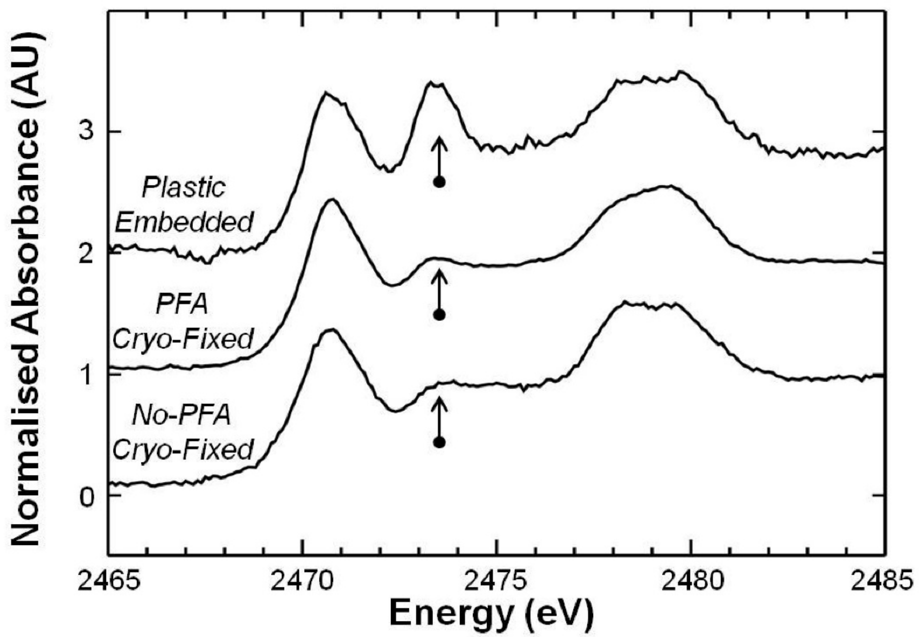


Hackett et al. (2015) ACS Chemical Neuroscience

SAMPLE PREPARATION – DON'T USE CHEMICAL FIXATION

Chemical fixation = sulfonic acid leaching & thiol oxidation
Paraffin or plastic embedding = massive sulfur oxidation





Hackett et al. (2012) ACS Chemical Neuroscience
Hackett et al. (2016) ACS Biochemistry

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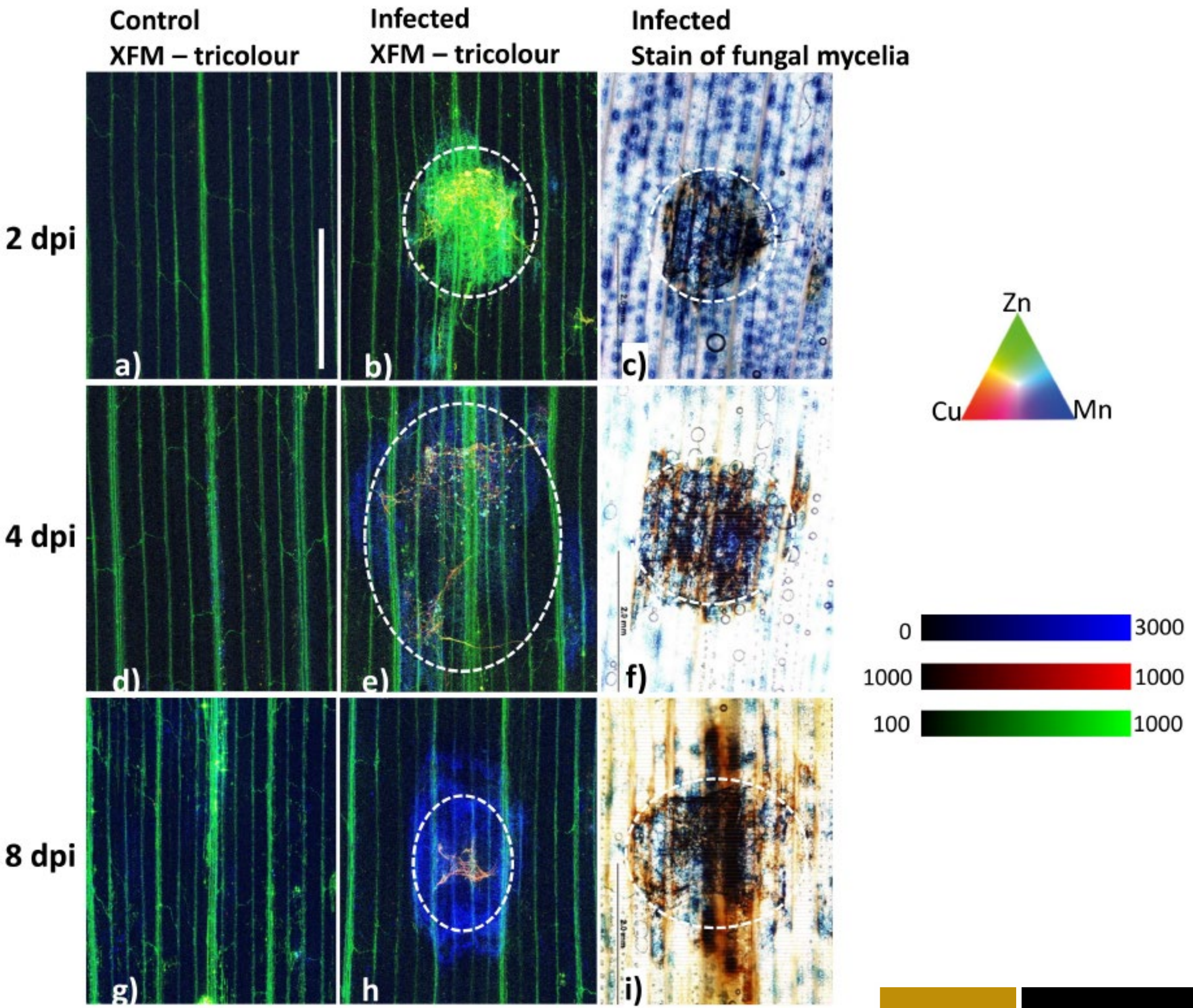
Australian Research Council

4. THE ROLE OF METALS IN WHEAT FUNGAL DISEASE



Slide courtesy of Fatima Naim and Mark Gibberd

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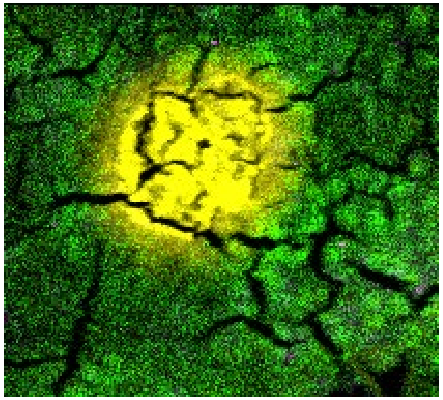
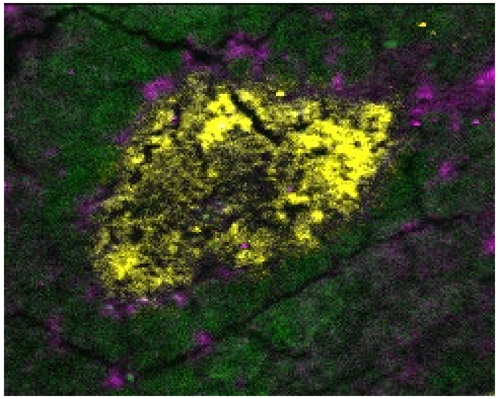
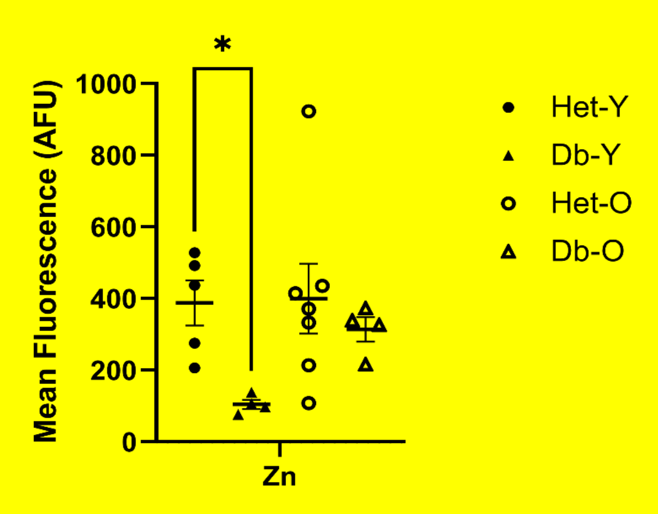
4. ZINC HOMEOSTASIS IN THE PANCREAS

Young (WT)



Young (dbdb)





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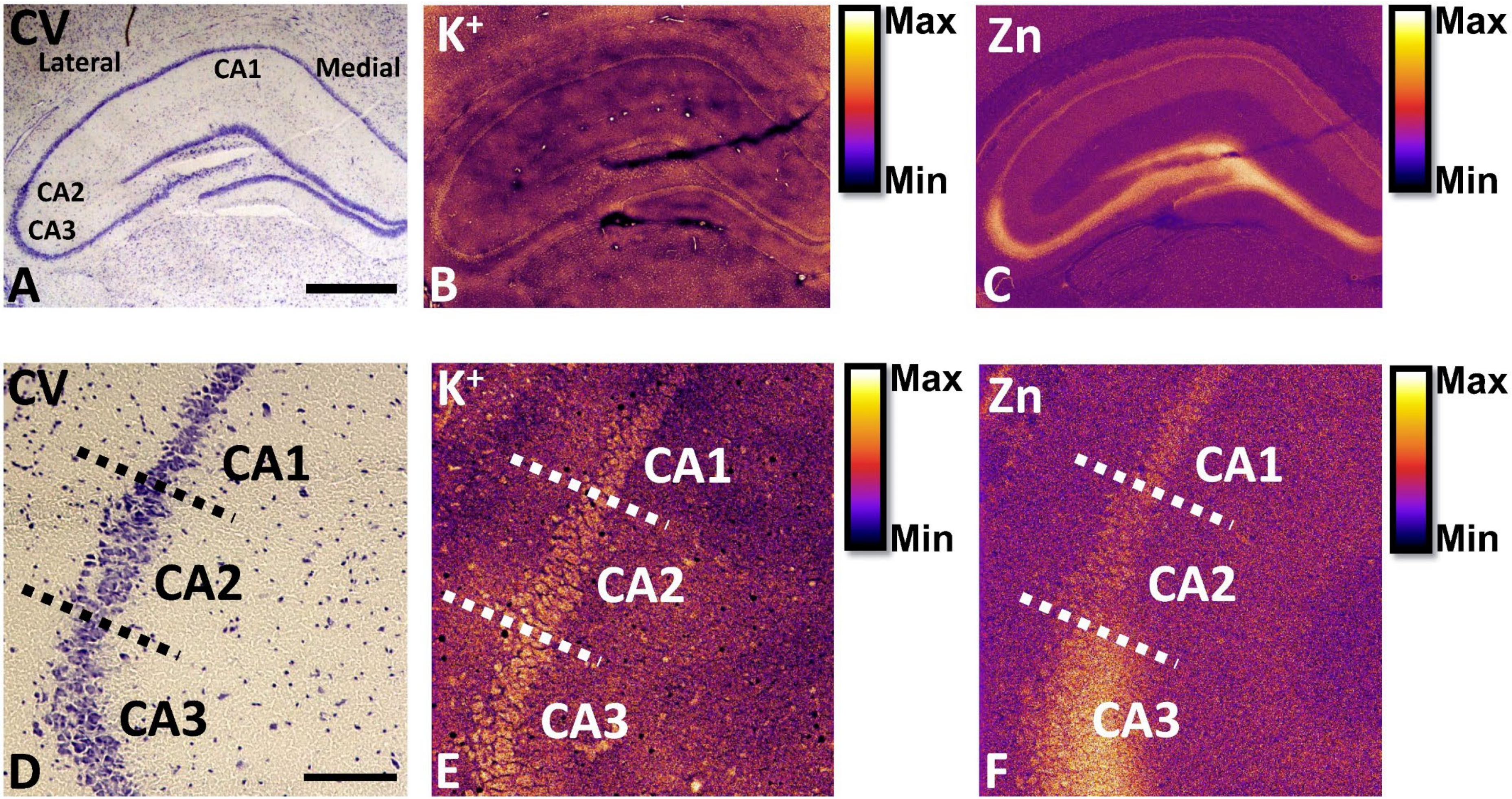
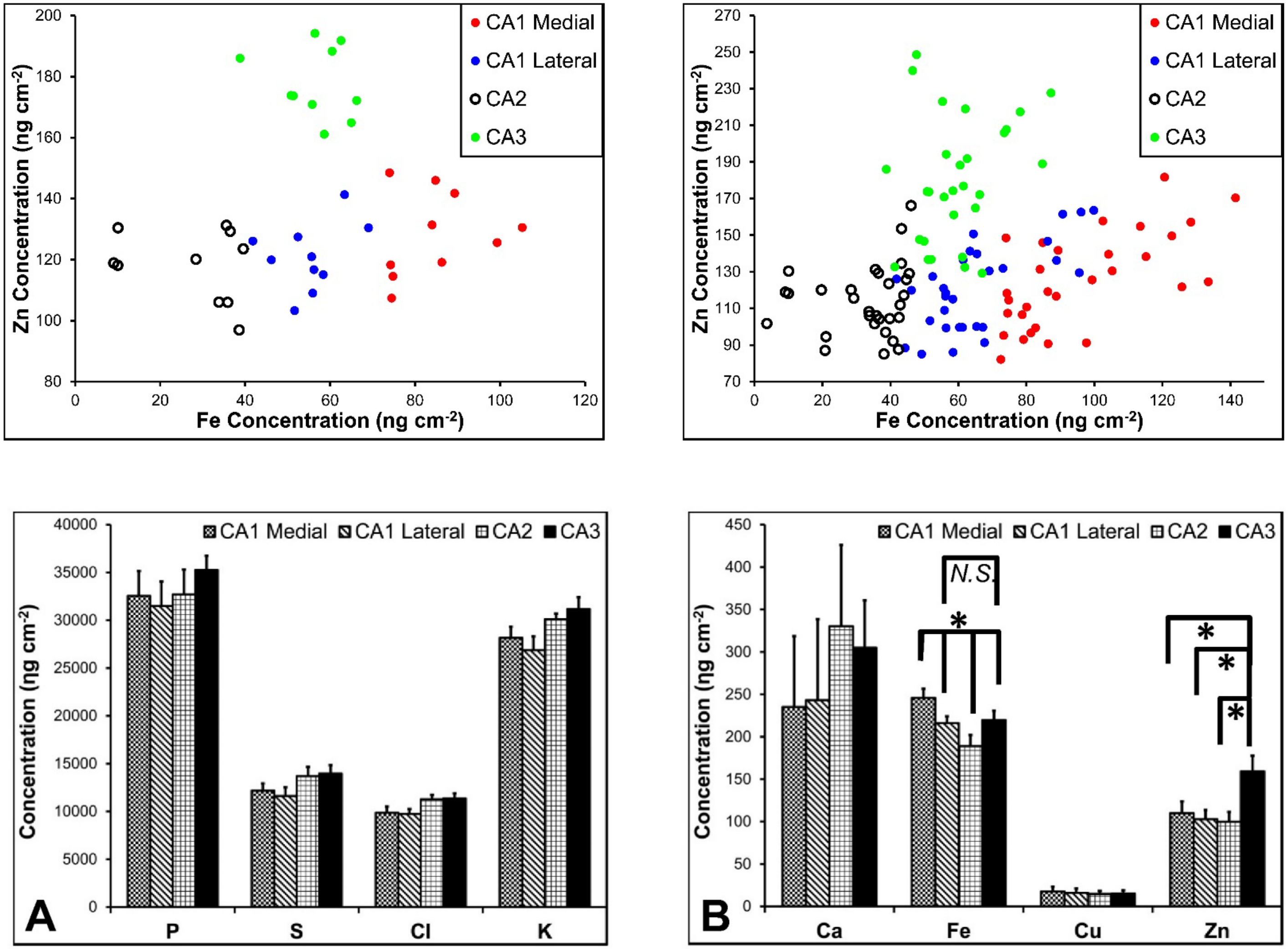
Slide courtesy of Gae Ellison

ANATOMICAL DEMARCATION FROM ELEMENTAL MAPS

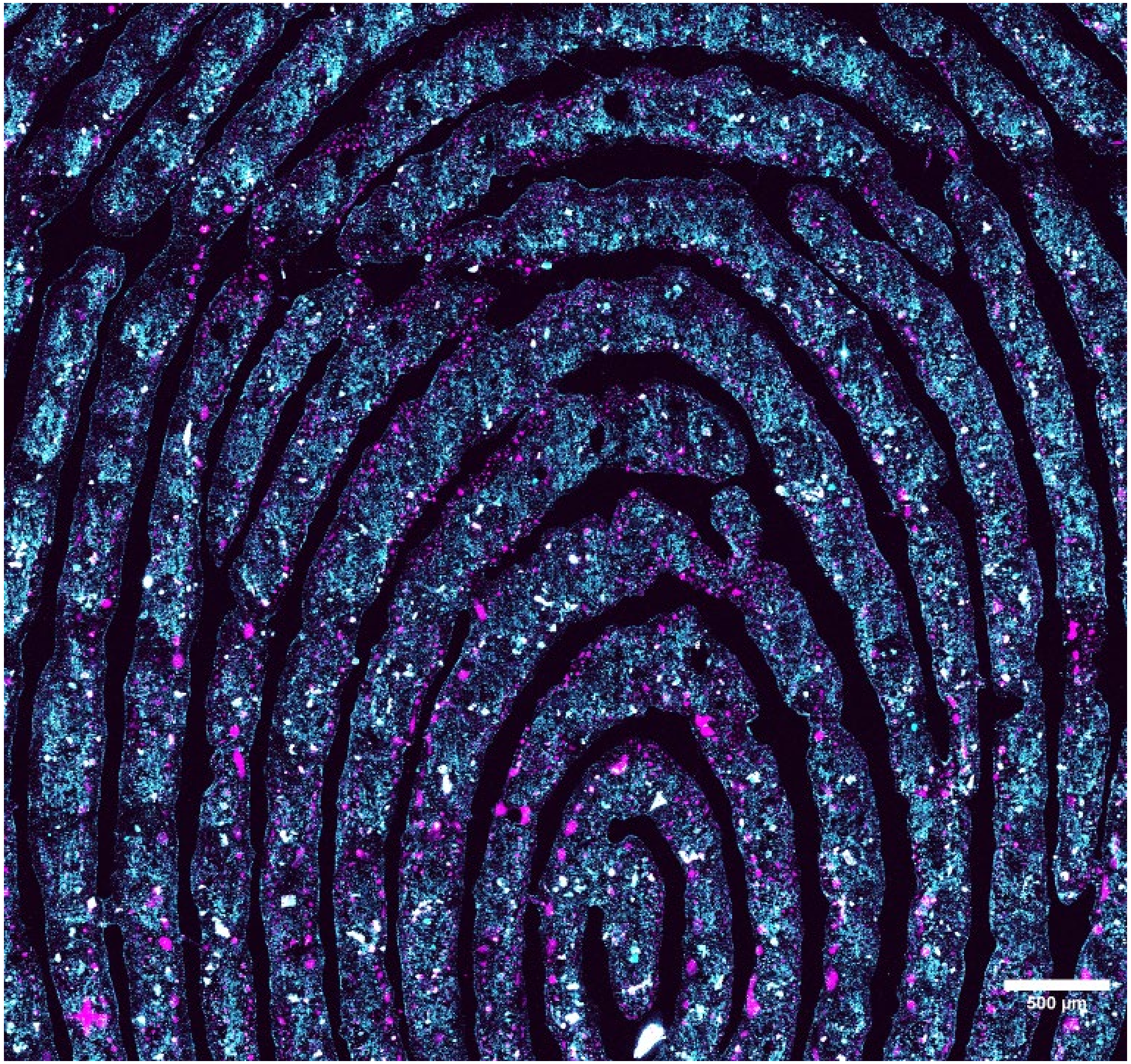


Hackett et. al. *Metallomics 11, 151-65, **2019***

2. DETERMINING ELEMENTAL PROFILES IN RAT HIPPOCAMPAL PYRAMIDAL NEURONS



4. ELEMENTAL MAPPING LATENT FINGERMARKS



X-ray Fluorescence Microscopy

Aim: Image the spatial distribution of elemental components within a natural fingermark deposit

Approach: 8 donors deposit natural fingermarks on silicon nitride slides or Mylar

Elements detected at trace levels:

Calcium, Chloride, Copper, Iron, Nickel, Potassium, Titanium , Zinc

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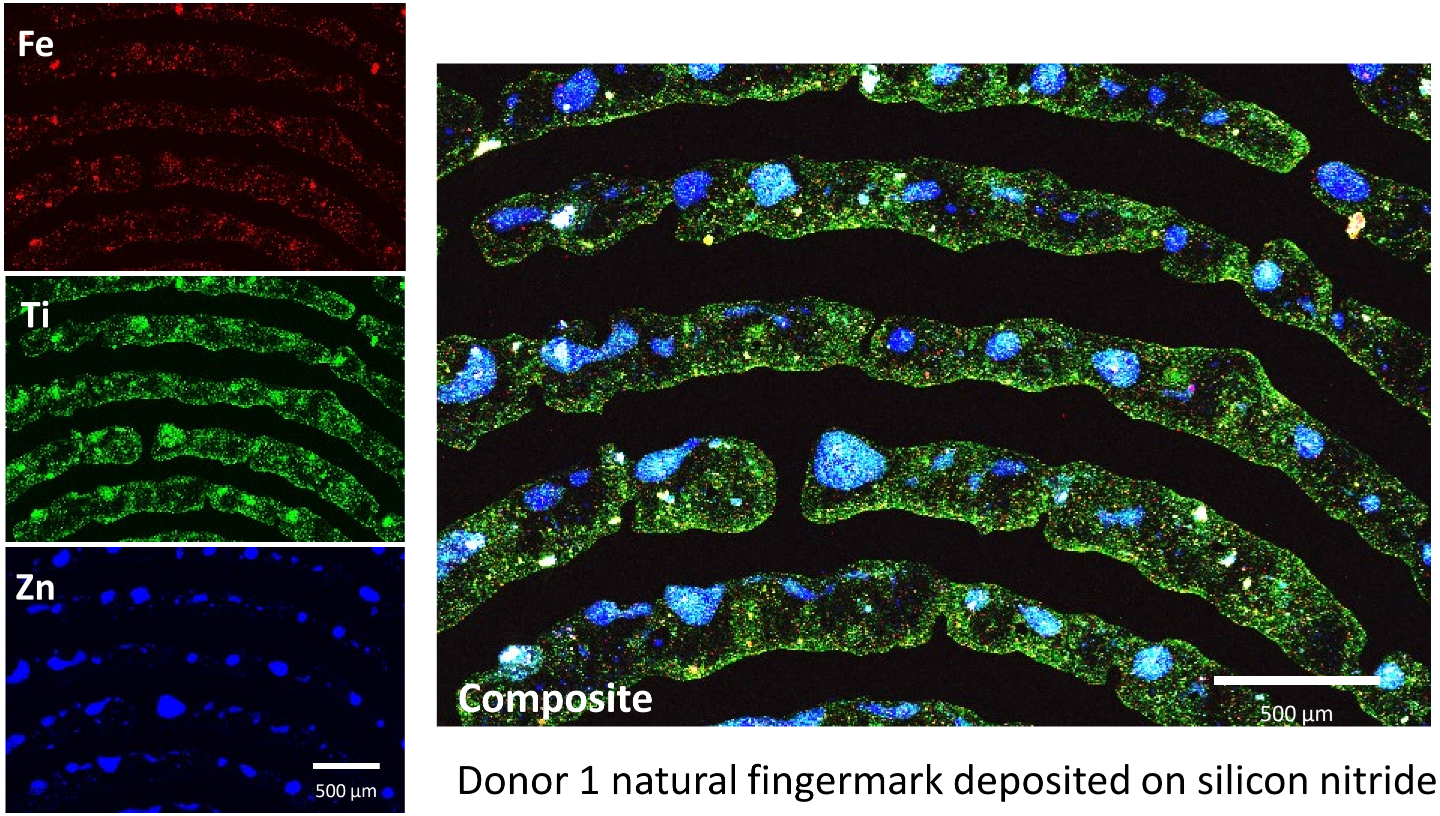
Slide courtesy of Rhiannon Boseley and Simon Lewis

4. Elemental mapping latent fingermarks

Co-localisation of metals from proposed exogenous sources



Donor 1 natural fingermark deposited on silicon nitride

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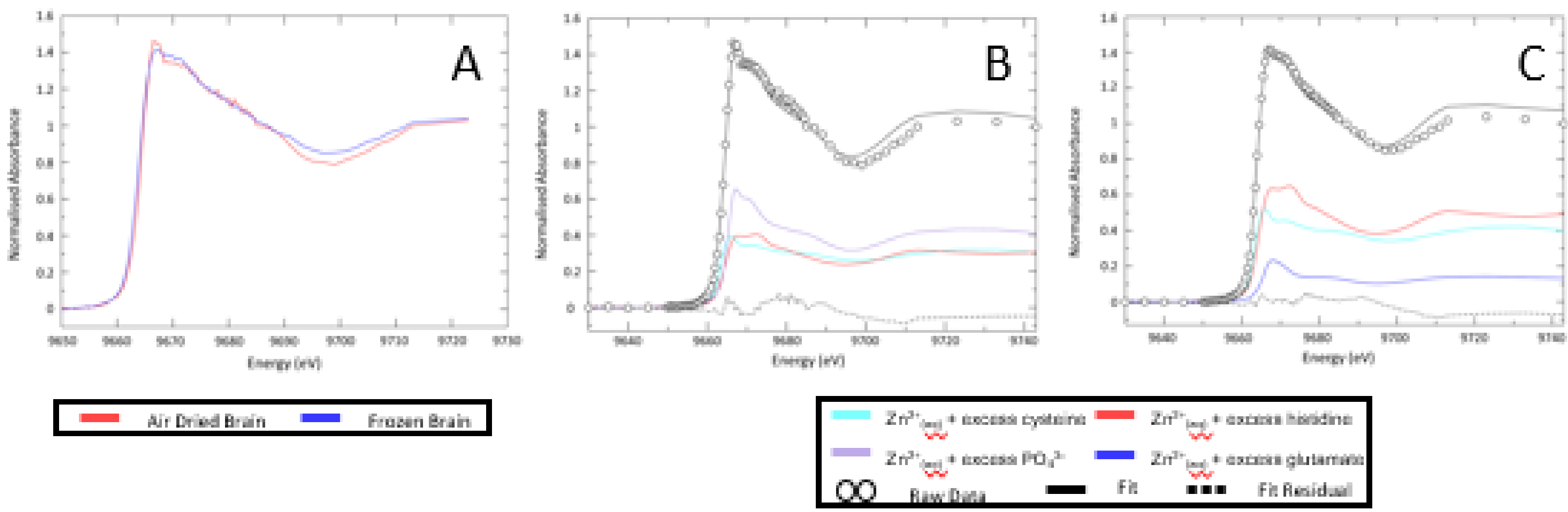
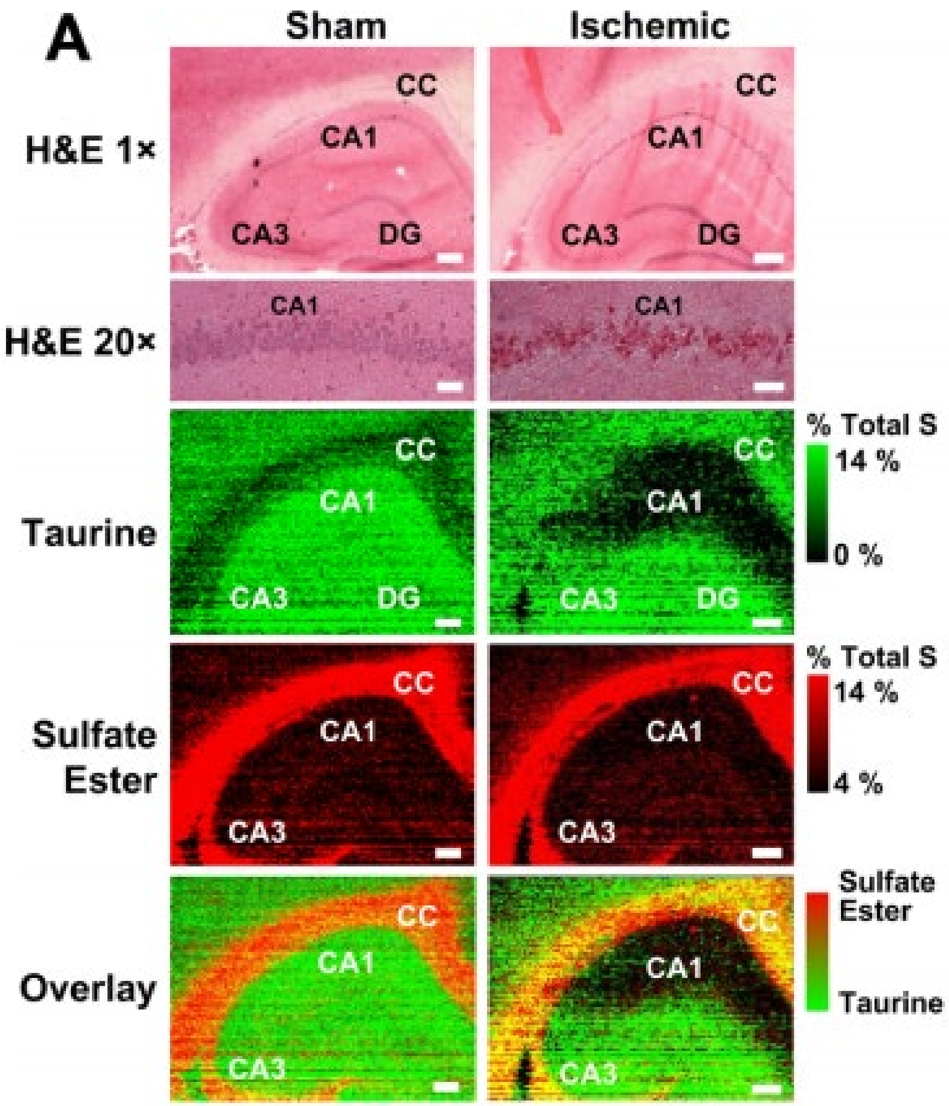
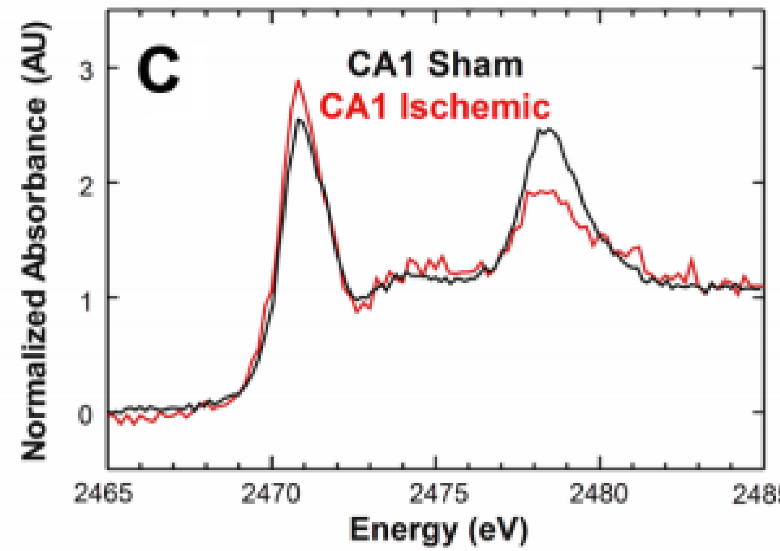
Slide courtesy of Rhiannon Boseley and Simon Lewis

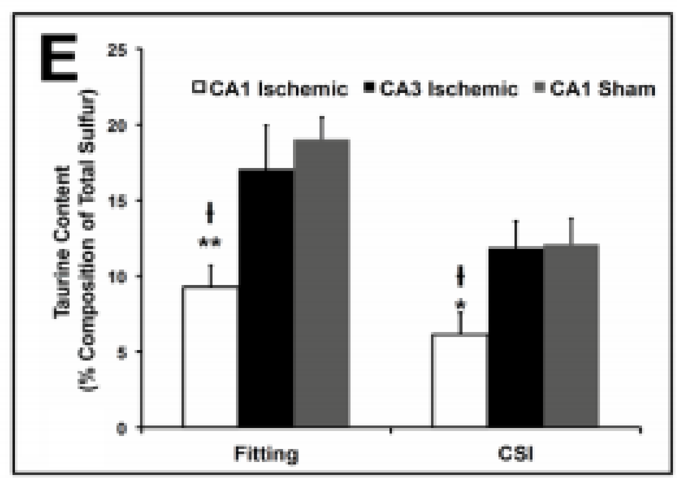
EFFECT OF AIR-DRYING



MEDIUM ENERGY X-RAY ABSORPTION SPECTROSCOPY







Hackett et. al. (**2015**) *Free Rad. Biol. Med.*, Hackett et. al. (**2016**) *Analytical Chemistry*. Hackett et al. (**2016**) *Biochemistry*