Application of SEM and epifluorescence for spatial localization of metals in mouse kidney tissue

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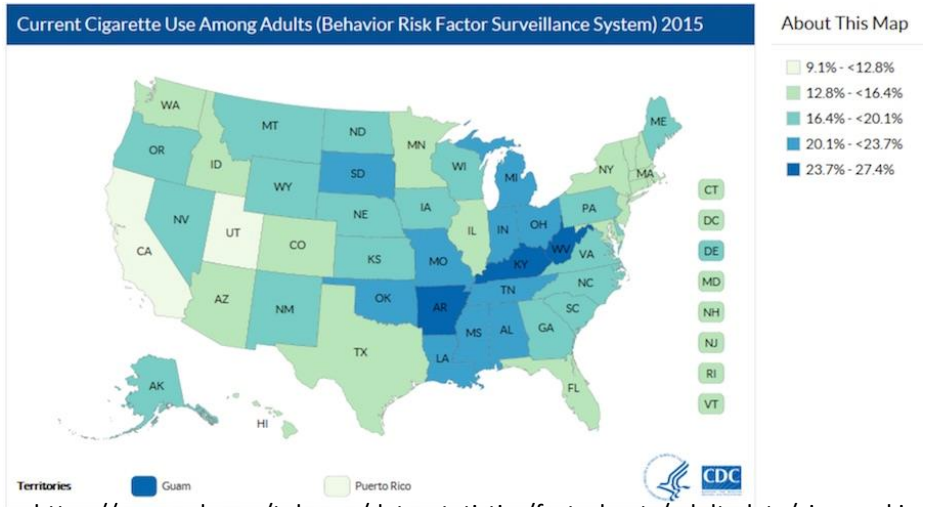
Kentuckian Heavy Metal Exposure - Smoking

Cadmium Exposure



https://www.cdc.gov/tobacco/data_statistics/fact_sheets/adult_data/cig_smoking/

Smoking

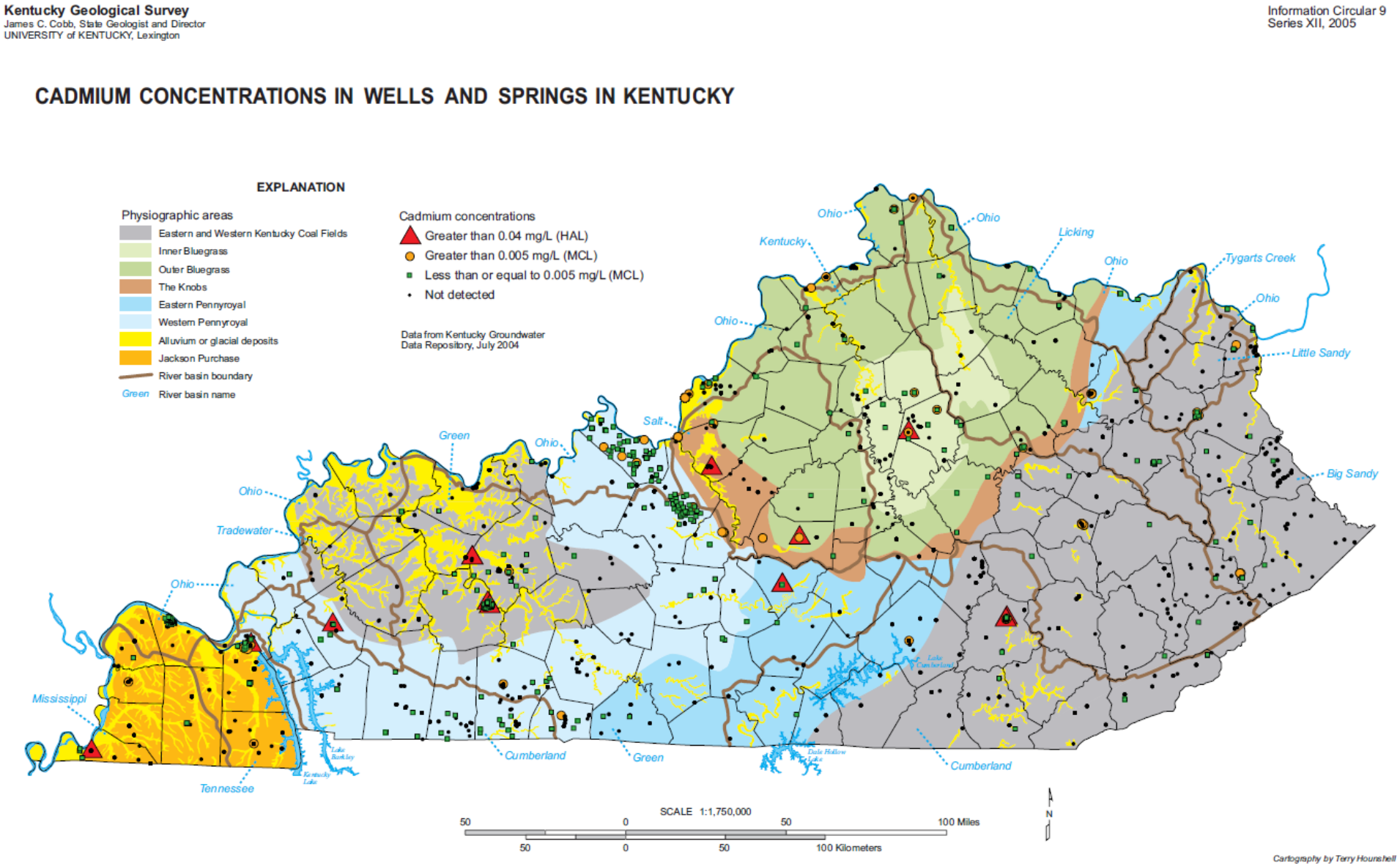
15.1 % of US population smokes cigarettes.
Kentucky is higher than national average: 23.7%-27.4%.

Diet

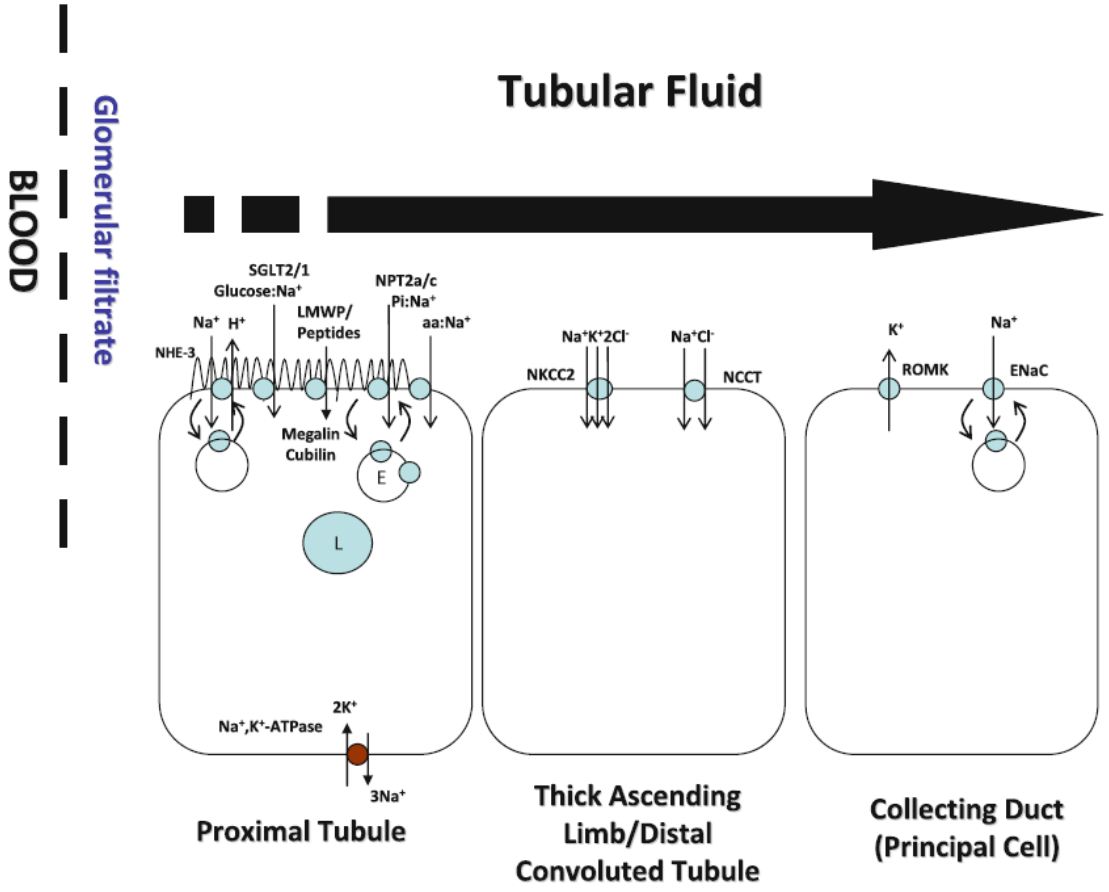
Non-smokers sequester Cd primarily through diet. Primary sources are bread, potatoes, shell-fish, and green leafy vegetables.

NHANES study found in Cd urine of 94-98% non-smokers and 95-99% smokers. (Tohoku J Exp Med. 2017;241(1):65-87)

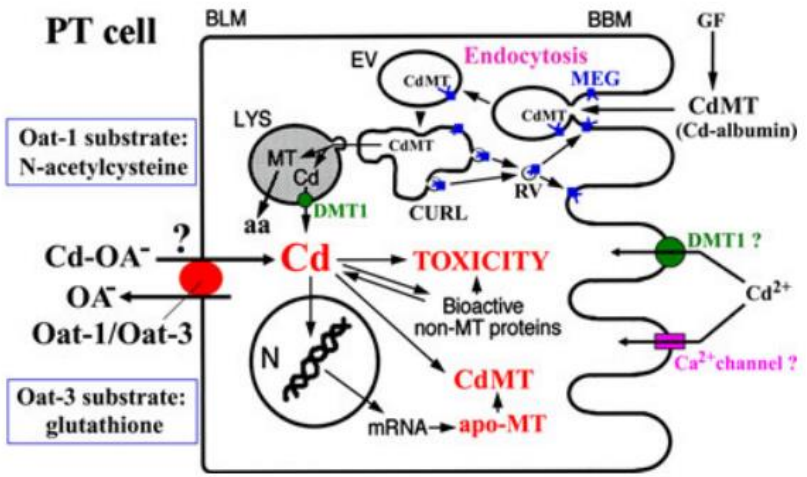
Kentuckian Heavy Metal Exposure - Groundwater



Cadmium causes kidney disease

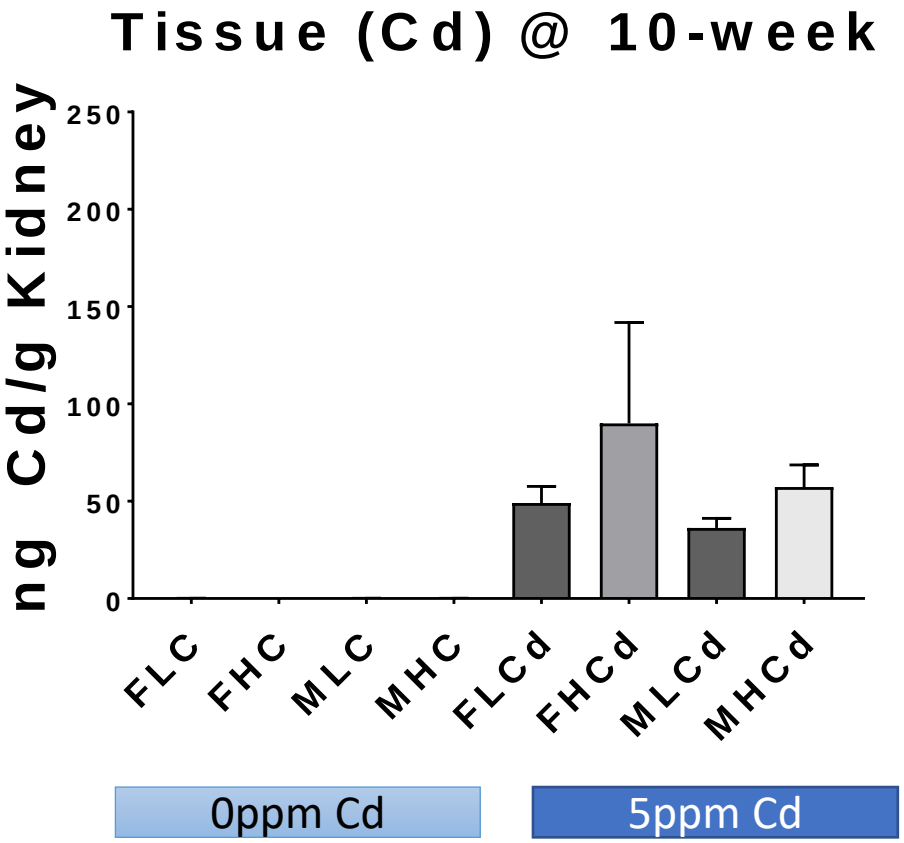


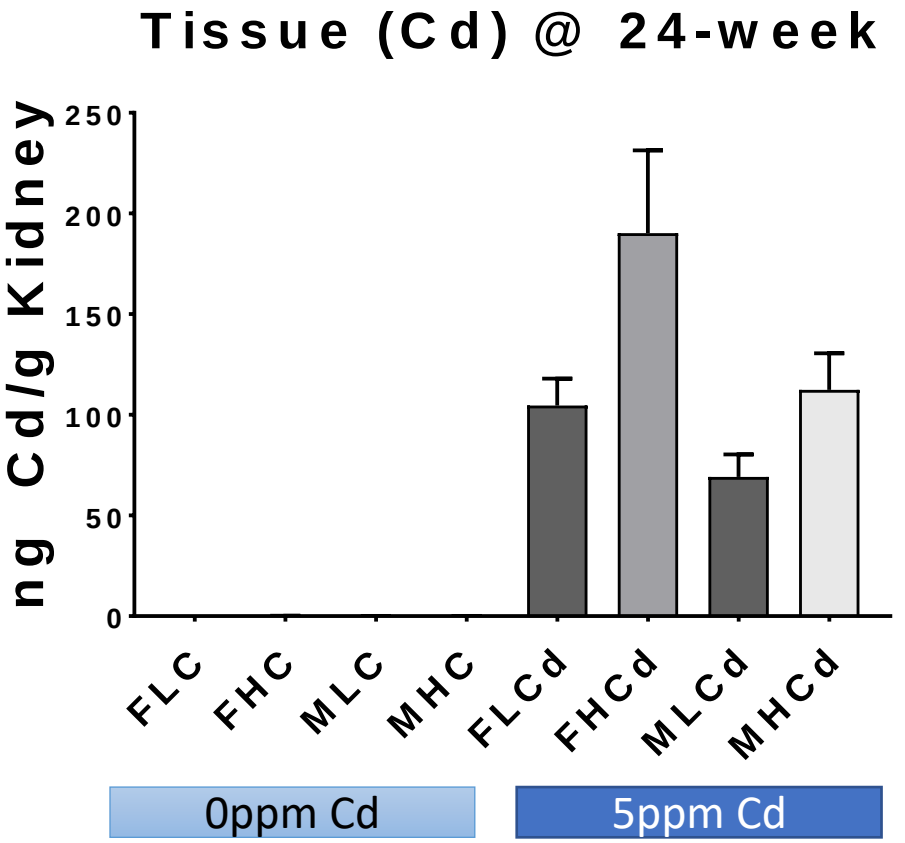
Johri N., Jacquillet G., and Unwin R., Biometals (2010) 23:783–792 Heavy metal poisoning: the effects of cadmium on the kidney.



Sabolic´ I etal., Biometals (2010) 23:897–926 Role of metallothionein in cadmium traffic and toxicity in kidneys and other mammalian organs

Cadmium, fat and renal tissue cadmium levels





Dr. Lu Cai's lab using ICP-MS; inductively coupled plasma-mass spectrometry.

Renal Cortex Histology

Histology:

PAS-stained kidney sections from Male and female mice:

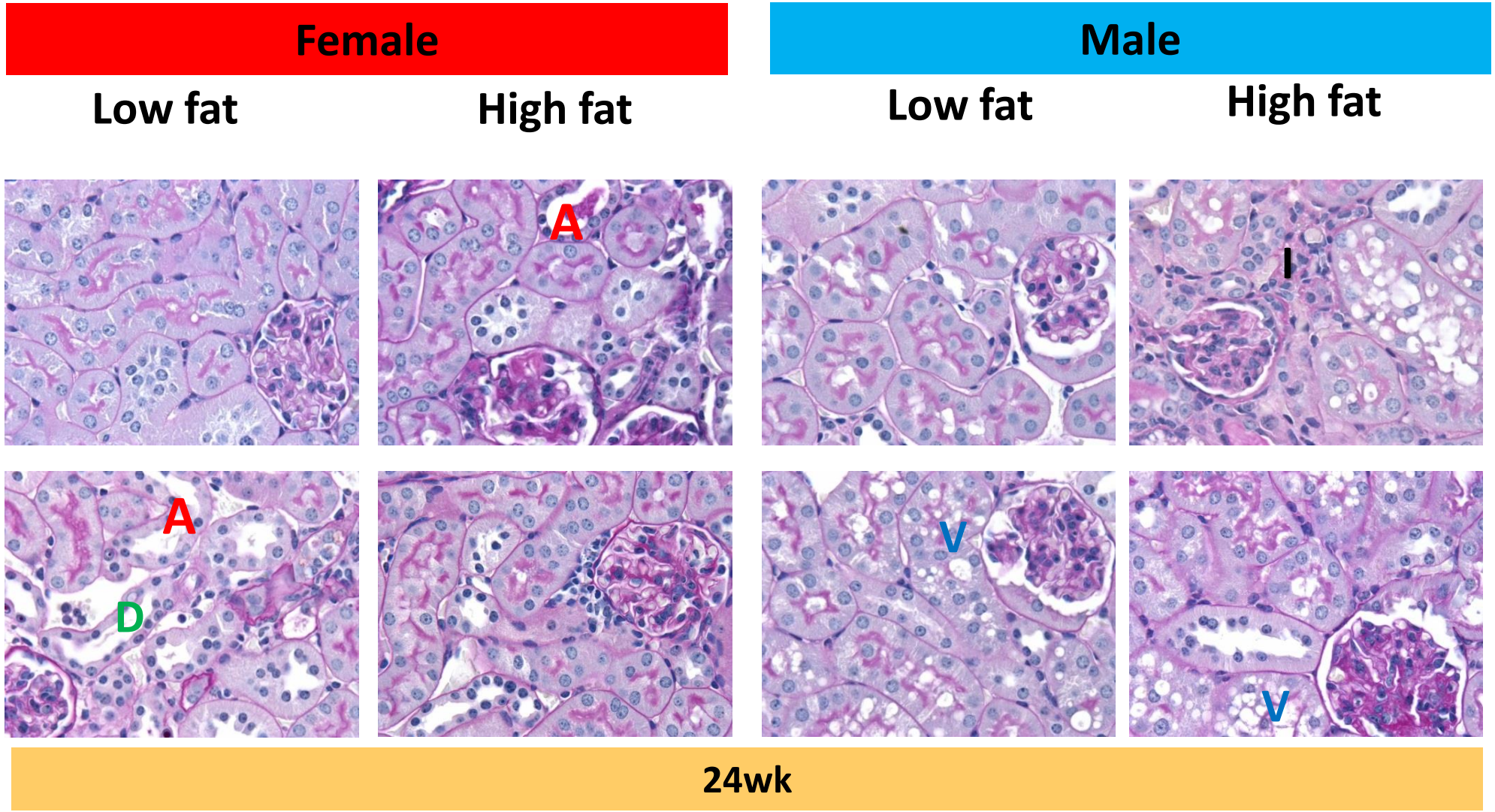
- 24 weeks of age
- low or high fat diet
- +/- 5ppm cadmium

A, tubule atrophy/dilation/casts

D, degenerative tubule cell toxicity

I, inflammatory foci

V, tubule vacuolization



Objective: Spatial localization of tissue metals levels using methodology that enables laser capture microdissection spectroscopy?

Table 1. Spatially Resolved Microanalytical Techniques for in Situ Imaging of Trace Metals in Biology[6−11]

analytical method	detection limit	spatial resolution (μm)	analytical depth (μm)	quantification
electron probe X-ray microanalysis (EPXMA)[6]	100−1000 μg/g	0.03	0.1−1	semiquantitative
proton beam microprobe (PIXE, RBS, and STIM)[6]	1−10 μg/g	0.2−2	10−100	quantitative (PIXE-RBS)
X-ray microprobe (SXRF, μXAS, μXANES)[6,7]	0.1−1 (SXRF), 100 (μXAS) μg/g	0.03−0.2	>100	quantitative
laser ablation−inductively coupled plasma−mass spectrometry (LA−ICP−MS)[6]	0.01 μg/g	15−50	200	semiquantitative
secondary ion mass spectrometry (SIMS)[6]	0.1 μg/g	0.05	0.1	quantitative
magnetic resonance imaging (MRI)[8]	mM to low μM	25−100	no limit	semiquantitative
positron emission tomography (PET)[8]	high pM	1000−2000	no limit	semiquantitative
autoradiography[9]	<0.01 μg/g	0.1	no limit	semiquantitative
autometallography[10,11]	nM	0.001−0.005 (EM)	0.01−1 (EM)	semiquantitative
optical fluorescence microscopy[8]	pM to nM	2000−3000 (in vivo), 0.2−0.5 (in vitro)	<1 cm	qualitative/ semiquantitative
visible light microscopy	low μM	0.2−0.5	0.01−1	qualitative

- **Study Objectives**: To determine the potential for spatial metals localization using energy dispersive X-ray analysis (EDXA or EDAX) with the Thermo Fisher Scientific Apreo C LoVac FESEM with high- and low-voltage ultra-high-resolution capabilities, back-scattered detector (BSD), scanning transmission electron microscopy (STEM) detector and energy dispersive X-ray spectroscopy (EDS) housed in the UofL Micro-Nano Technology Center (MNTC).

McRae R et al *In situ* imaging of metals in cells and tissues *Chem. Rev.* **2009,** *109,* 4780–4827

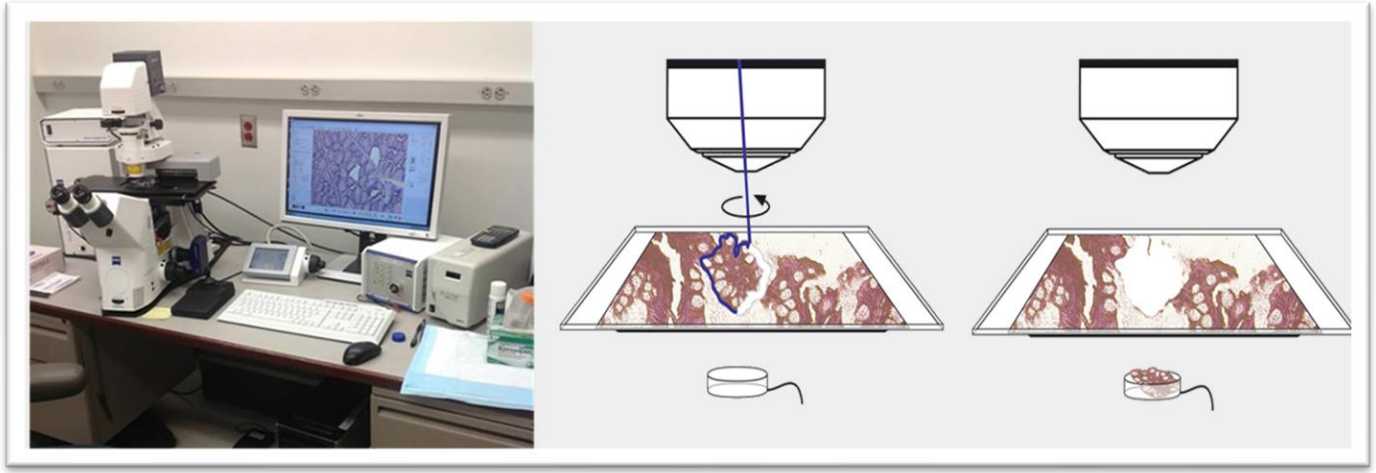
Data collected and analyzed

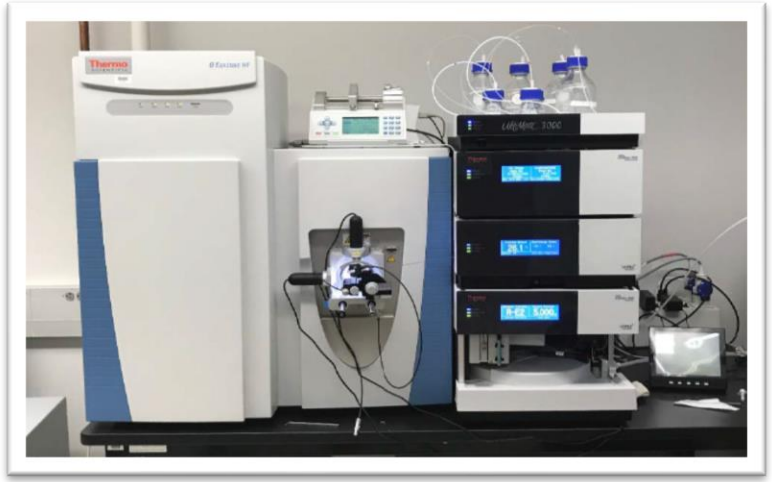
- **Hypothesis**: Spatial metals analysis of mouse kidney tissue using EDXA would identify tissue zonal regions enriched in cadmium (Cd++) or arsenic (As) in mice with whole life exposure to either 5ppm cadmium (Cd++) or 100ppb arsenite.

- **Experimental Design**:
  - Kidney tissue from female mice having whole life exposure to (a) 5ppm Cd or (b) 100ppb As was be prepared for SEM EDAX analysis.
  - Prior inductively coupled plasma-mass spectrometry (ICP-MS) studies prioritized kidneys from female mice fed high fat diets and metals
    - (eg median Cd++ levels of 190±40ng Cd++/g kidney tissue).

Spatially resolved proteomics

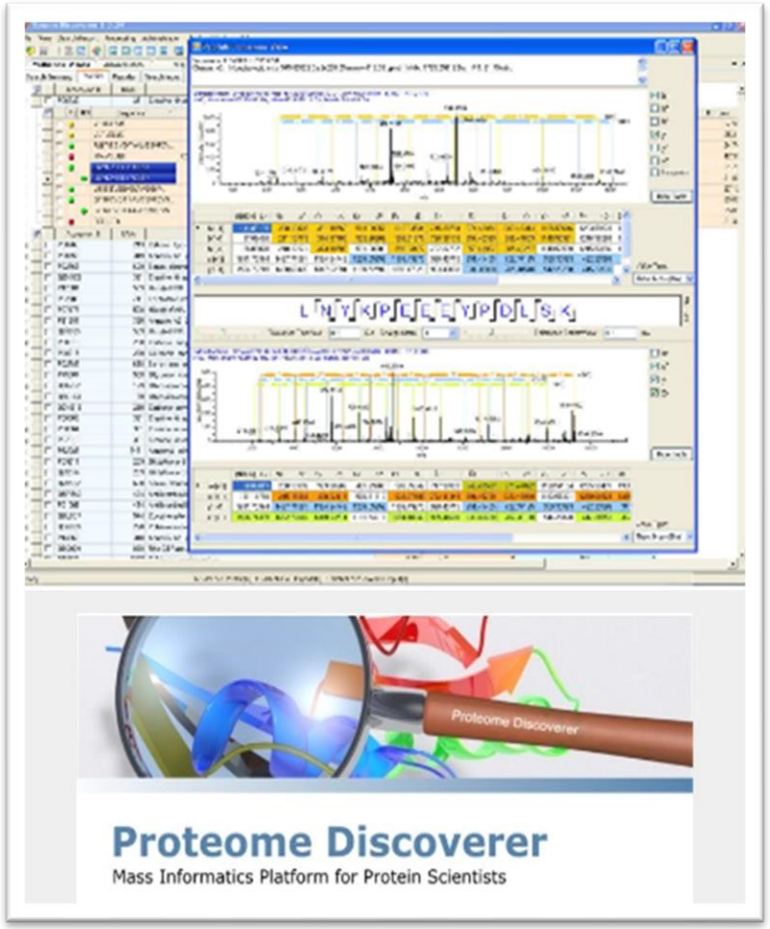
Isolate tissue based on spatially resolved information



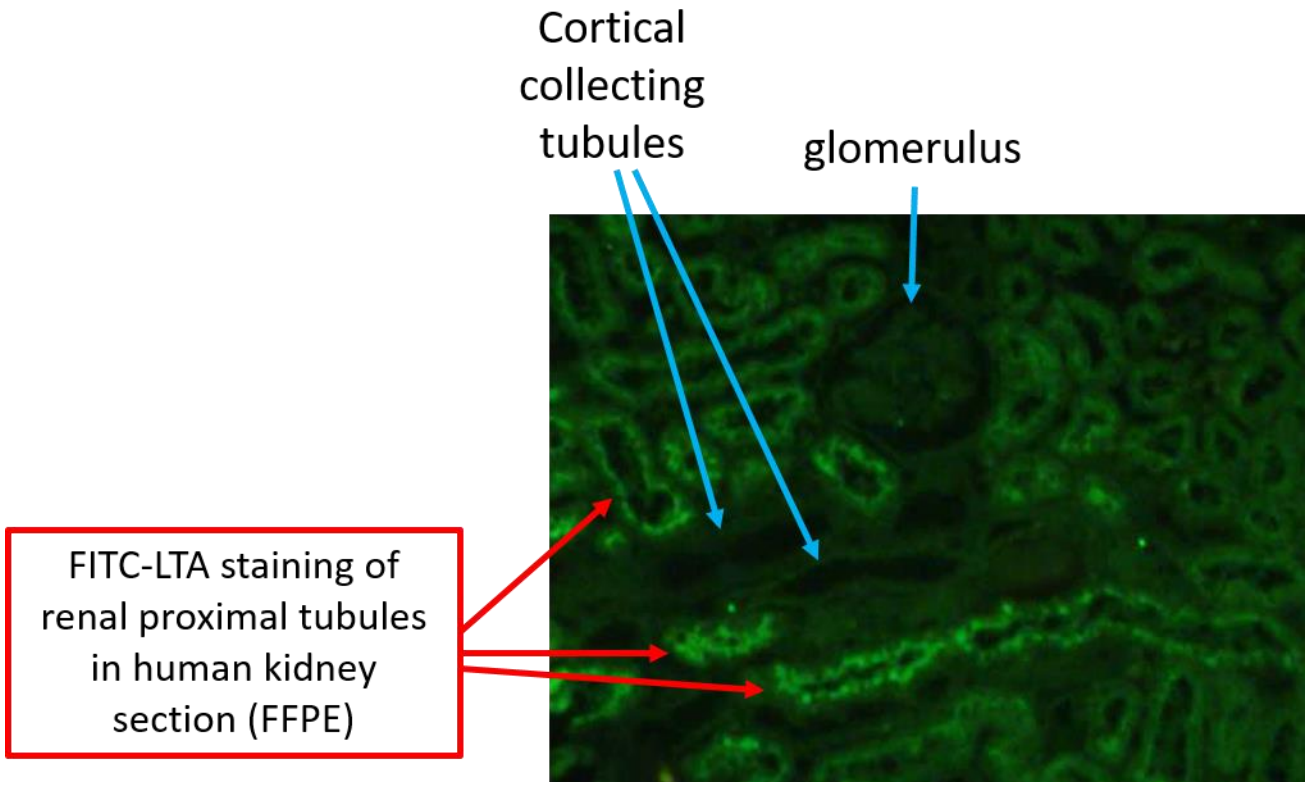


Collect “OMICS” Data

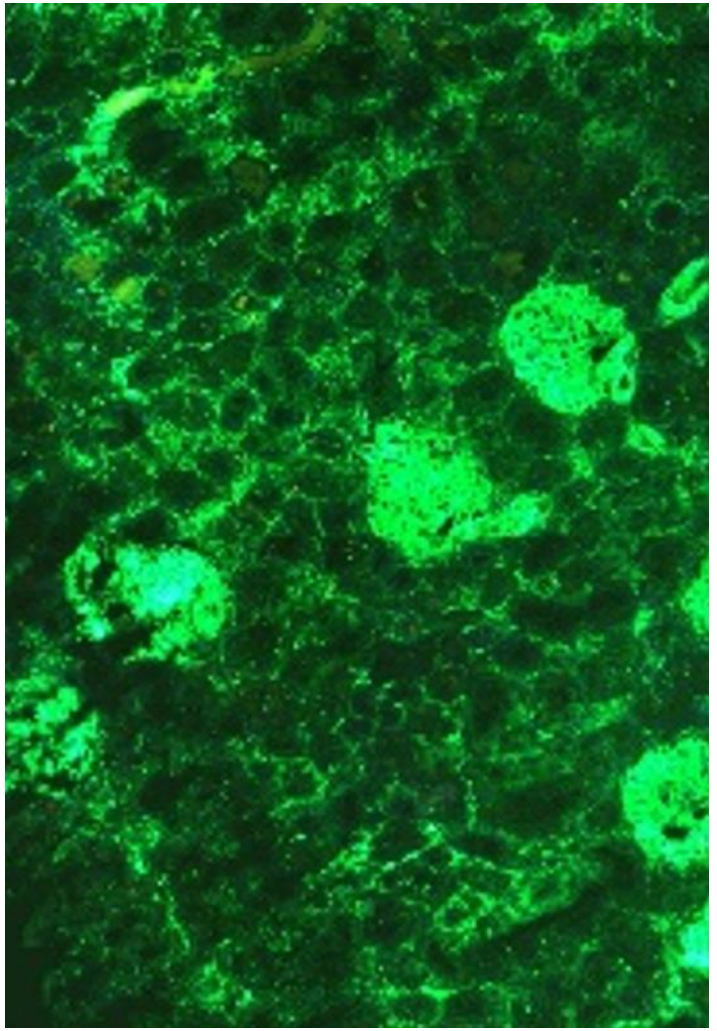
Informatics to interpret “OMICS” Data



Spatial resolution using tissue based histologic data and visual or fluorescent stains

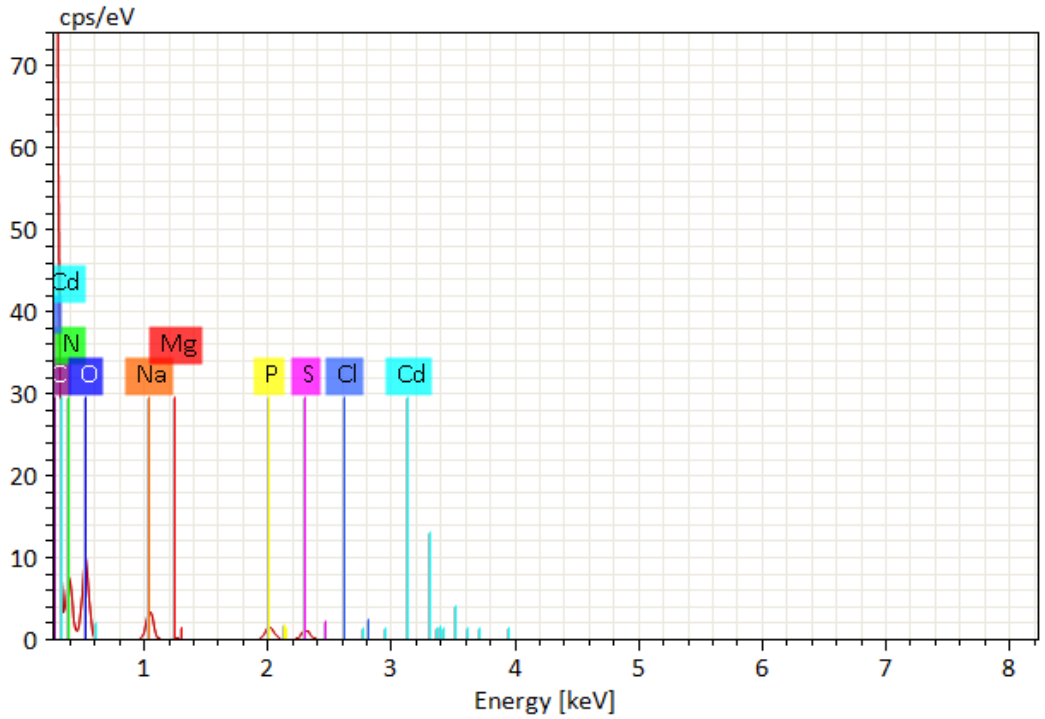


FITC-Phalloidin staining of human kidney section (Frozen)



Microscopy core director Michelle Barati, PhD (michelle.barati@louisville.edu)
Leica LMD 6500 Laser Capture Microdissection Microscope (fee-for-service).

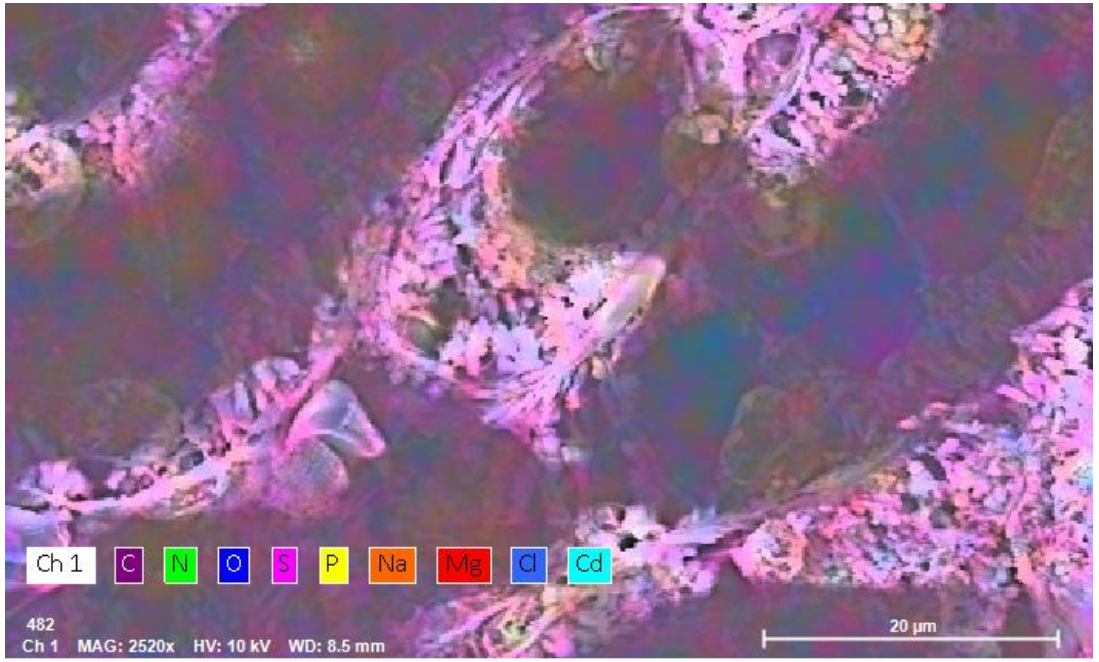
Spectral and compositional characteristics of metals selected for study

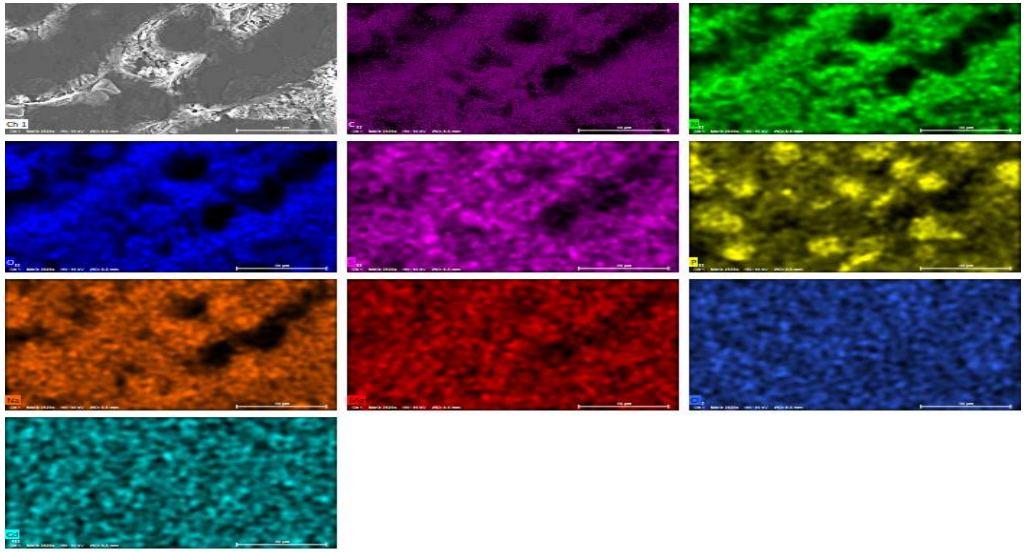


Map

Element	At. No.	Netto	Mass [%]	Mass Norm. [%]	Atom [%]	abs. error [%] (1 sigma)	rel. error [%] (1 sigma)
C	6	2220659	66.76	66.76	72.02	2.37	3.54
N	7	116496	17.76	17.76	16.43	0.88	4.96
O	8	177390	12.50	12.50	10.13	0.57	4.58
S	16	31977	0.71	0.71	0.29	0.02	2.55
P	15	43547	0.86	0.86	0.36	0.02	2.61
Na	11	71716	1.28	1.28	0.72	0.06	4.54
Mg	12	3816	0.06	0.06	0.03	0.00	8.08
Cl	17	2930	0.08	0.08	0.03	0.01	6.85
Cd	48	0	0.00	0.00	0.00	0.00	1.30
		Sum	**100.00**	**100.00**	**100.00**		

Female mouse kidney
24 week exposure to 5ppm Cd in drinking water.





Name	Date	Time	HV [kV]	Mag	WD [mm]
482	3/7/2022	10:48:16 AM	10.0 keV	2520x	8.5 mm

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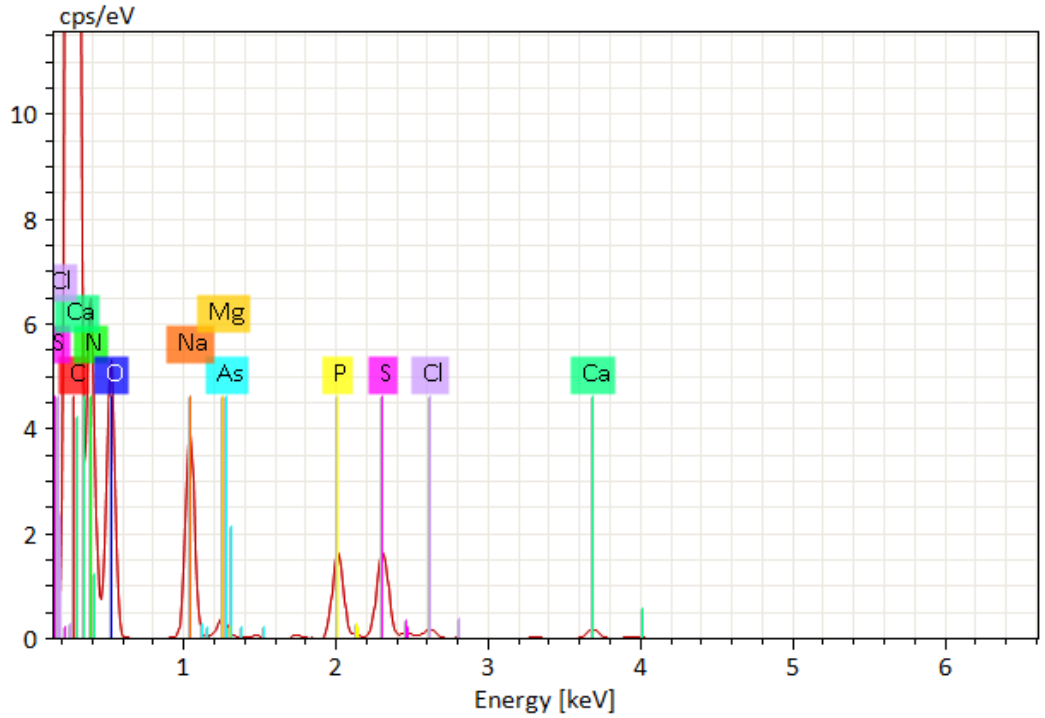
3/7/2022	10:48:16 AM	10.0 keV	2520x	8.5 mm

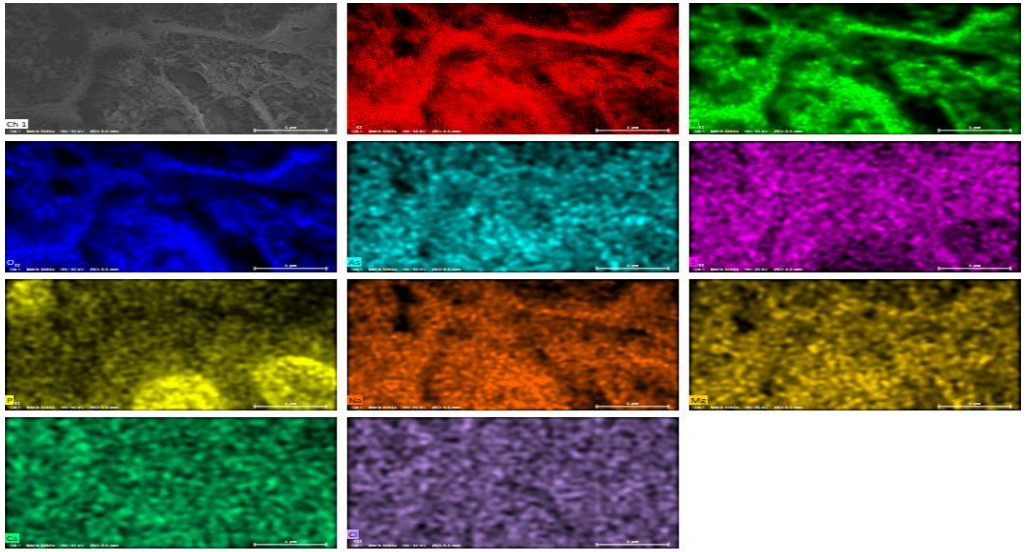
Spectral and compositional characteristics of metals selected for study



Map

Element	At. No.	Netto	Mass [%]	Mass Norm. [%]	Atom [%]	abs. error [%] (1 sigma)	rel. error [%] (1 sigma)
C	6	3502732	77.88	77.88	82.94	2.80	3.60
N	7	69420	9.05	9.05	8.26	0.47	5.20
O	8	164081	8.33	8.33	6.66	0.38	4.58
As	33	4298	0.07	0.07	0.01	0.01	17.92
S	16	81909	1.25	1.25	0.50	0.03	2.22
P	15	75859	1.04	1.04	0.43	0.03	2.41
Na	11	134557	1.72	1.72	0.96	0.08	4.39
Mg	12	10675	0.12	0.12	0.06	0.01	6.88
Ca	20	9941	0.36	0.36	0.11	0.01	3.35
Cl	17	9147	0.17	0.17	0.06	0.01	3.87
		Sum	**100.00**	**100.00**	**100.00**		

Female mouse kidney
24 week exposure to 100ppb As in drinking water.



Name	Date	Time	HV [kV]	Mag	WD [mm]
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Date	Time	HV [kV]	Mag	WD [mm]
3/7/2022	10:18:49 AM	10.0 keV	8063x	8.5 mm

Issues encountered

- Tissue preparation- multiple steps that may have affected tissue quality
- Tissue sections placed onto silicon wafers were prepared using snap-frozen/embedded (5µm sections) and formalin-fixed paraffin embedded (FFPE, 10µm sections)
  - OCT removal: frozen tissue sectioned onto silicon wafers were thawed, rinsed with 0.1M phosphate buffered saline and dehydrated with two 20 minute equilibrations in a solution of 1:2 hexamethyldisilazane (HMDS): anhydrous ethanol followed by two 20 minute equilibrations in 100% HMDS. The dehydrated tissue sections on silicon wafers were left in the 100% HMDS solution covered or capped loosely in a fume hood overnight. The samples were imaged the following morning.
  - FFPE sections were evaluated with and without deparaffinization. All methods reveals substantial slide charging and very poor image quality.

Conclusions

SEM-*epifluorescence* microscopy and spatial localization of metals

- Fit for purpose, prospective tissue collection design essential.
- The current method can be applied to high abundance elements such as phosphorus
- Higher energy X-ray induced fluorescence approaches are needed for lower abundant target elements.

Acknowledgement
Thanks to…

- Julia Aebersold and Michael Martin, UofL Nano Micro Technology Center.
- KY-INBRE EM Pilot Grant funded through the National Institutes of Health National Institute of General Medical Sciences (5P20GM103436-22)
  - Michael Merchant (PI)
  - Michelle Barati (Co-I)