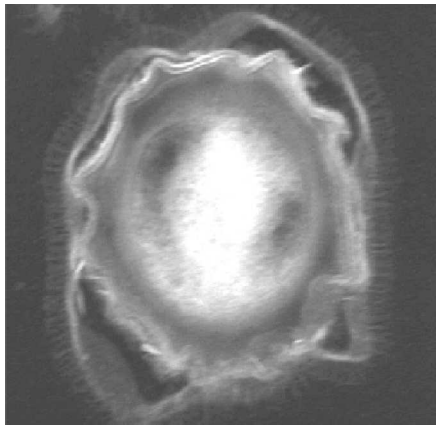
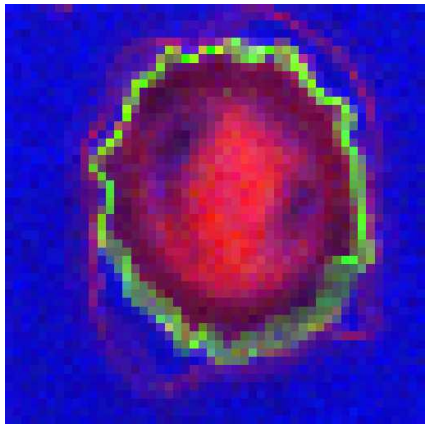




SAND2009-6205C

Elemental Microanalysis of *Bacillus Anthracis* Spores from the Amerithrax Case

Joseph R. Michael and Paul G. Kotula

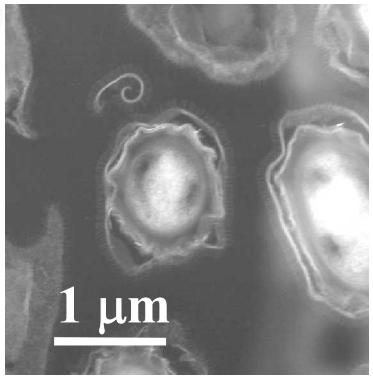
Materials Characterization Department 1822
Sandia National Laboratories, Albuquerque, NM 87185

Sandia is a multi-program laboratory operated by Sandia Corporation, a Lockheed Martin Company, for the United States Department of Energy under contract DE-AC04-94AL85000.

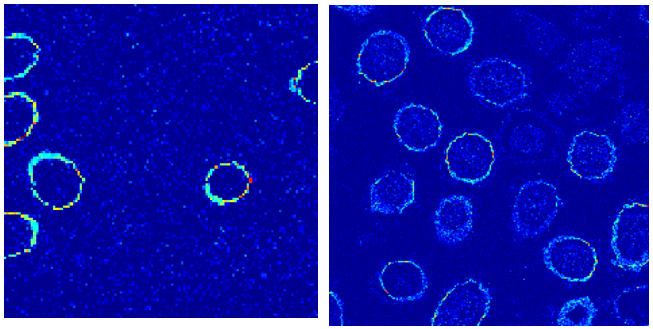
Outline

- Tools for elemental microanalysis
- Spectral imaging
- Microanalysis of Leahy and NYP with SEM
- Microanalysis of Leahy, NYP and Daschle with STEM and TOF-SIMS
- Are the letter powders unique with respect to elemental signatures?
- Summary

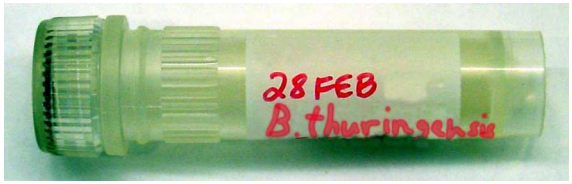
Signature Statistics



•Signals from Individual Spores

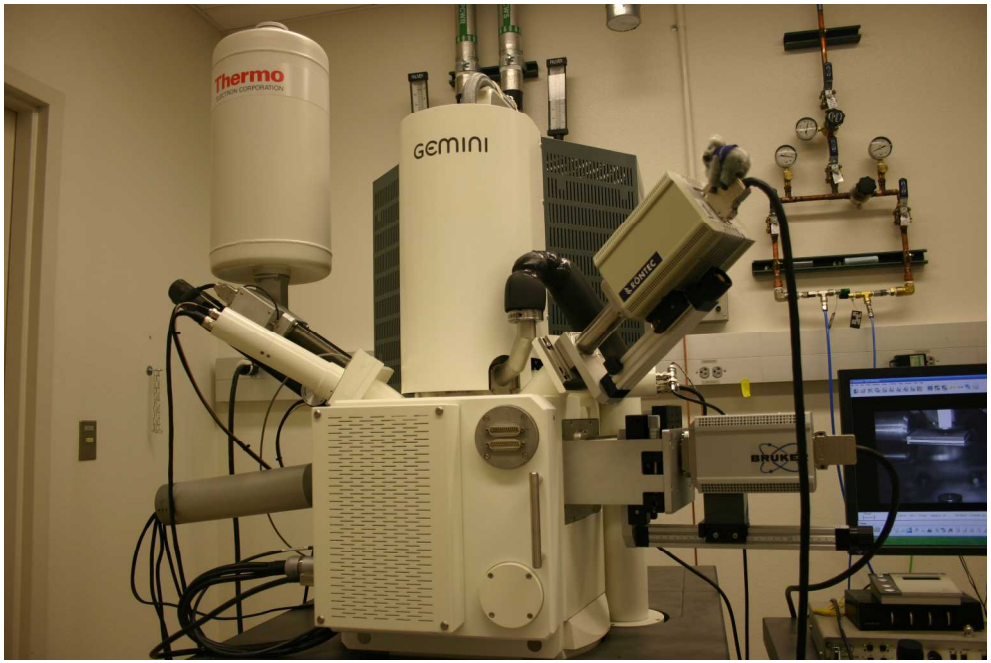


•Variability between fields of view

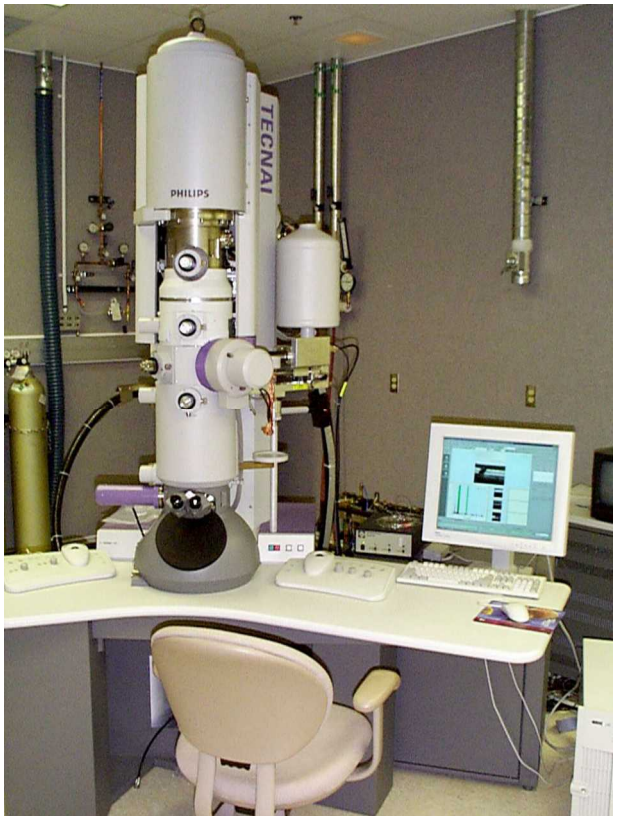


•Variability within bulk material

Comparison of SEM and STEM



SEM – scanning electron microscope



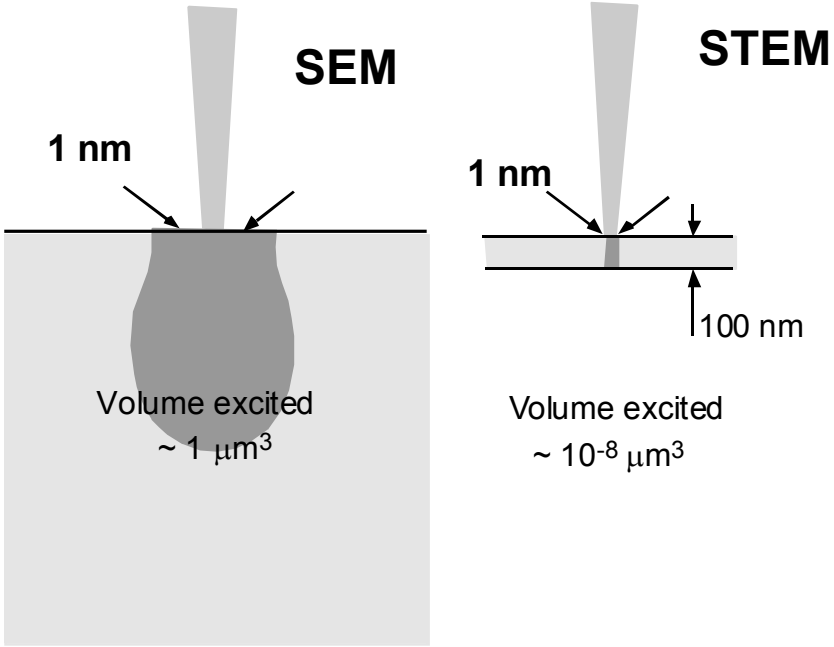
STEM – scanning transmission electron microscope

Comparison of SEM and STEM

SEM

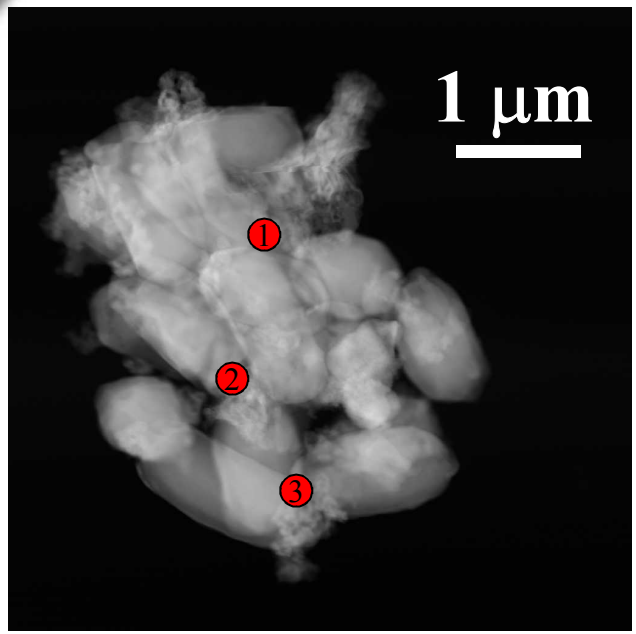
- Imaging – 0.6 nm currently
- Microanalysis – about 1 μm
- Elements – limited to >Be
- Diffraction for crystallography
- No sample preparation may be required

- **STEM**
- High Resolution Imaging – 0.2 nm
- Microanalysis – 1-2 nm spatial resolution
- Elements – limited to >Be
- Diffraction for crystallography
- Electron transparent (thin) samples



In this study we make use of the characteristic x-rays generated by the electron/sample interactions.

Automated Spectral-Image Analysis: Why?



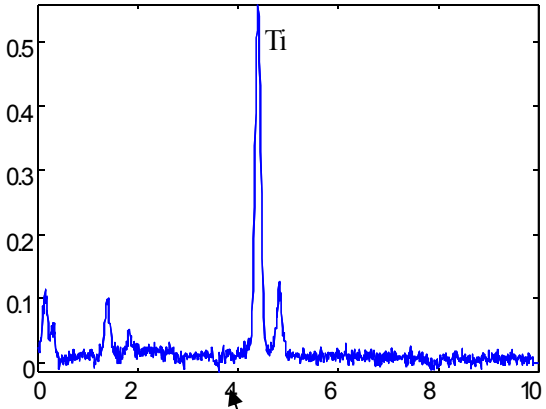
STEM image of spores

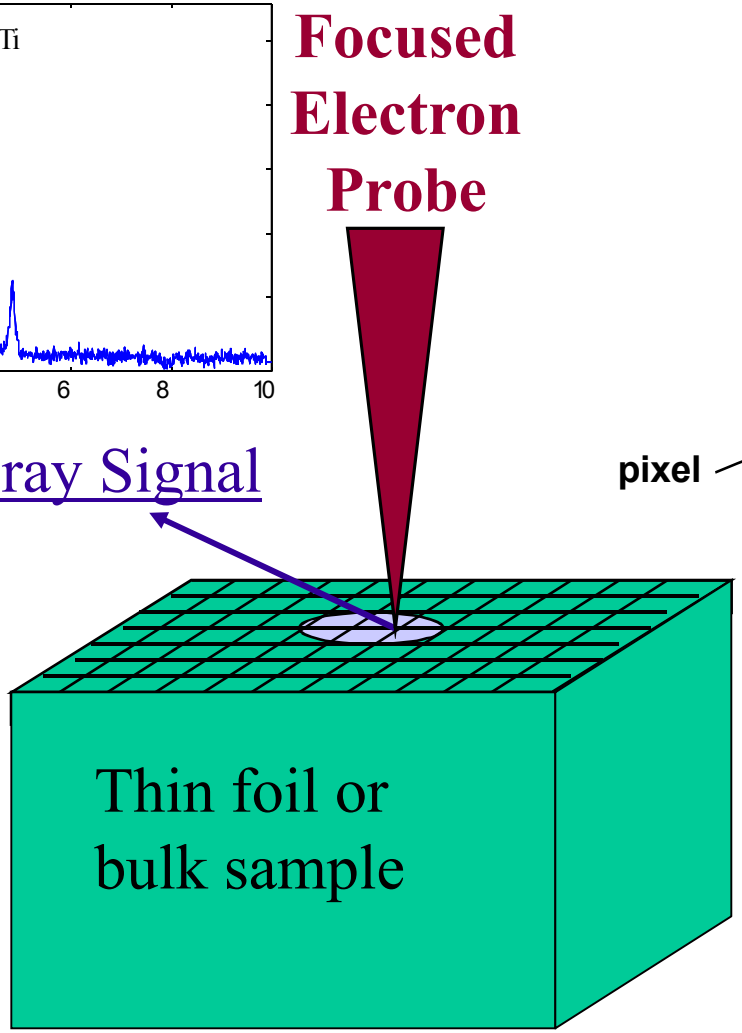
- **How do you comprehensively survey the chemistry of large sample areas?**
- **Point analyses can be subjective– where to take them from and how many.**
- **2D distributions of chemical phases are needed but simple mapping alone is not the answer. Mapping has potential artifacts and requires fore-knowledge.**

'**Chemical component images**' are needed–a spectrum from each component and an image describing where in the microstructure it's found

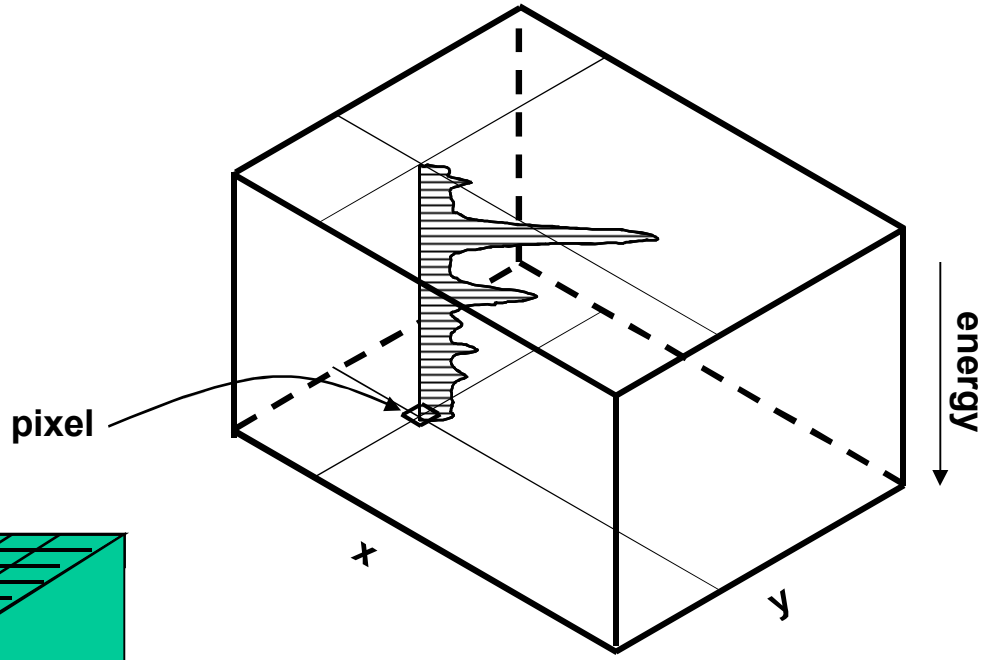
What are x-ray spectral images?

X-ray spectrum: chemical information from sample





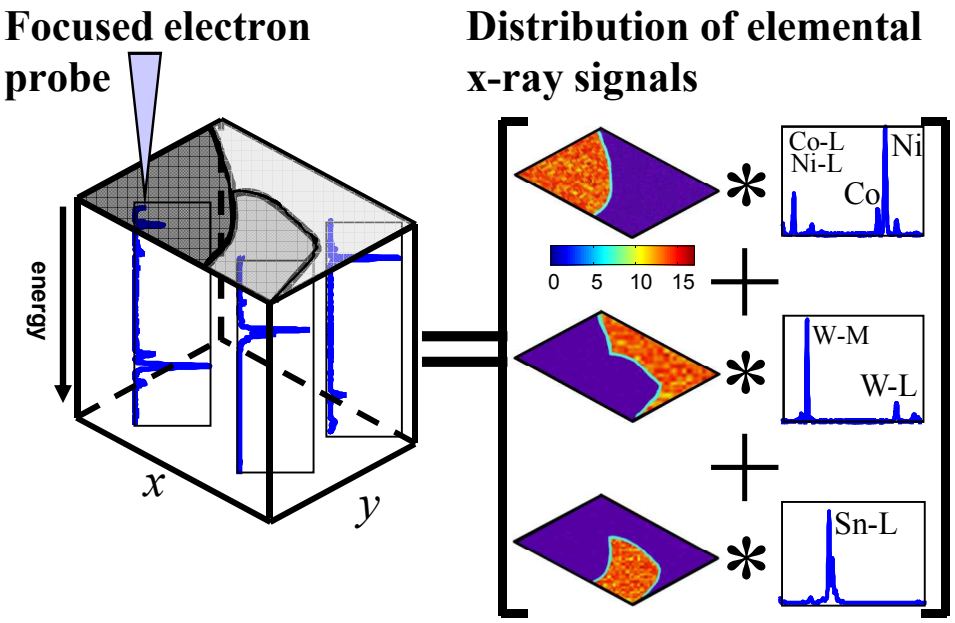
Spectral Image Data Set



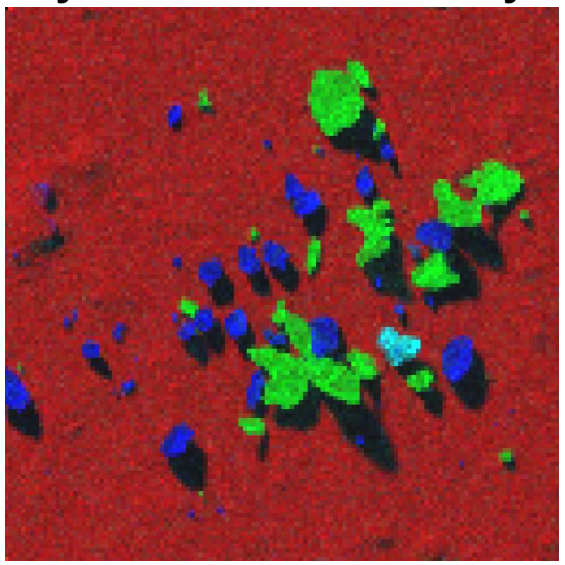
What do we do with all that data? Typically 10's of millions of pieces of data

Spectrum imaging for elemental forensic signatures

Spectrum imaging Statistical Analysis Tools



- Rapid decomposition of huge data sets
- Unbiased—no input guesses needed
- Elemental associations shown
- Ability to find “needle in haystack”



Red = C-support
Green = alumina
Blue = FeCo
Cyan = Ca-S-Si-O
Black = shadowed support

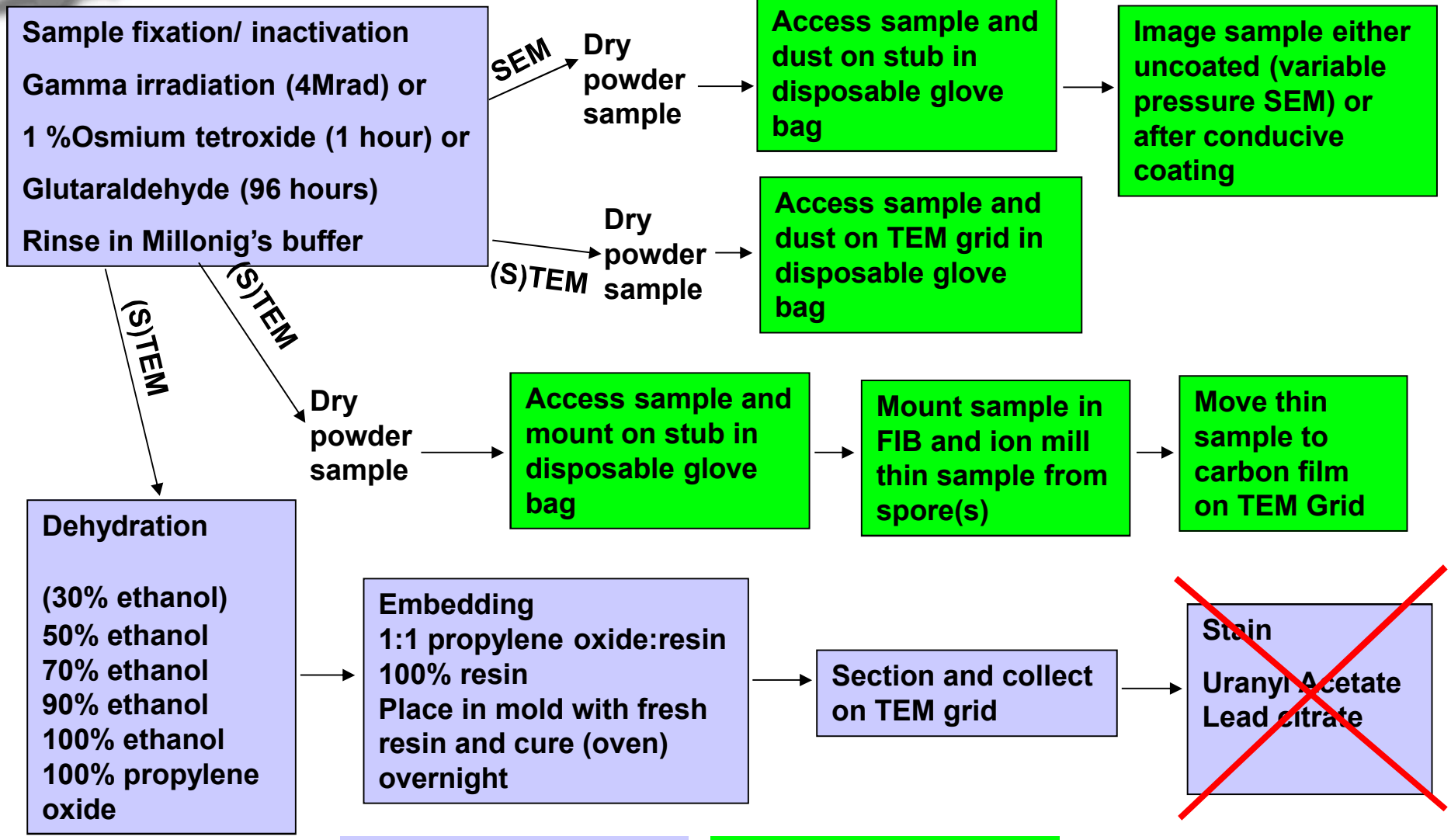
Keenan, M. R., and Kotula, P. G.,(2003) Apparatus and System for Multivariate Spectral Analysis., US Patent #6584413. (filing date June 1, 2001).

Keenan, M. R., and Kotula, P. G.,(2004) Method of Multivariate Spectral Analysis., US Patent #6675106. (filing date June 1, 2001)

Kotula, P. G., Keenan, M. R., Michael, J. R. (2003), “Automated Analysis of SEM X-ray Spectral Images: A Powerful New Microanalysis Tool, *Microscopy and Microanalysis*; Feb. 2003; vol.9, no.1, pp.1-17.

Sandia National Laboratories

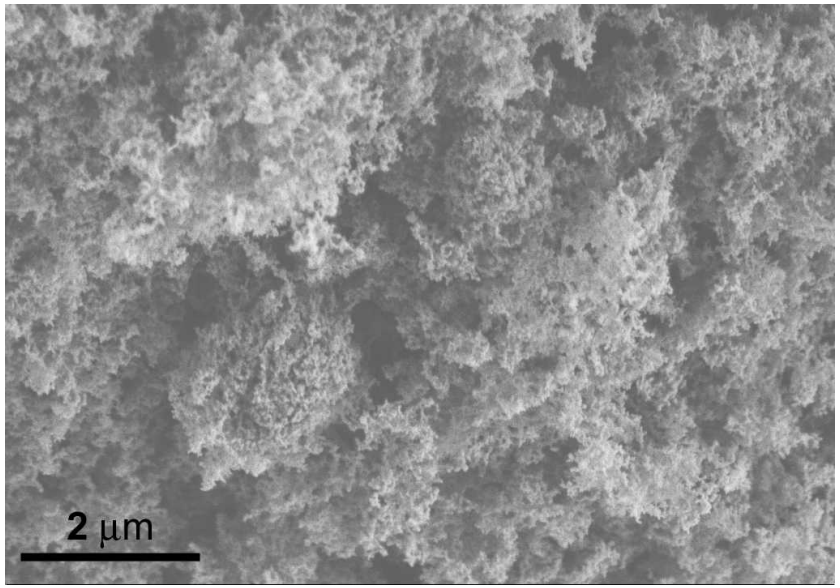
Preparation of samples for STEM or SEM



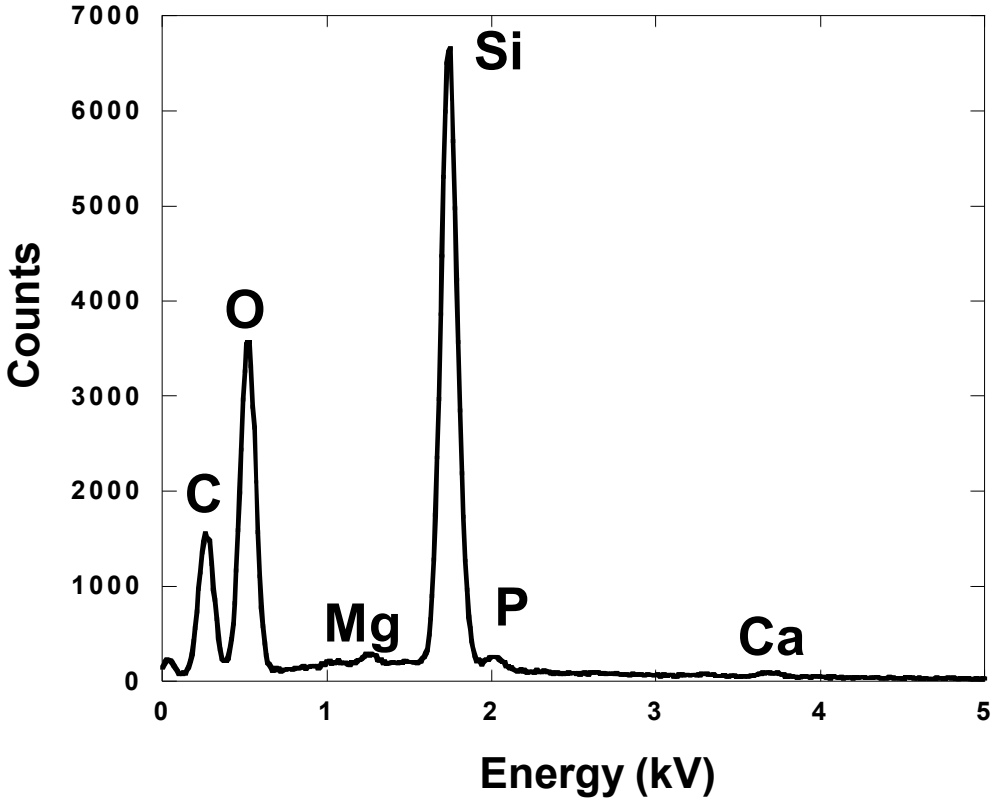
National Nuclear Security Administration

Sandia National Laboratories

Bacillus Thuringiensis treated with Silica nano-particles for flow improvements

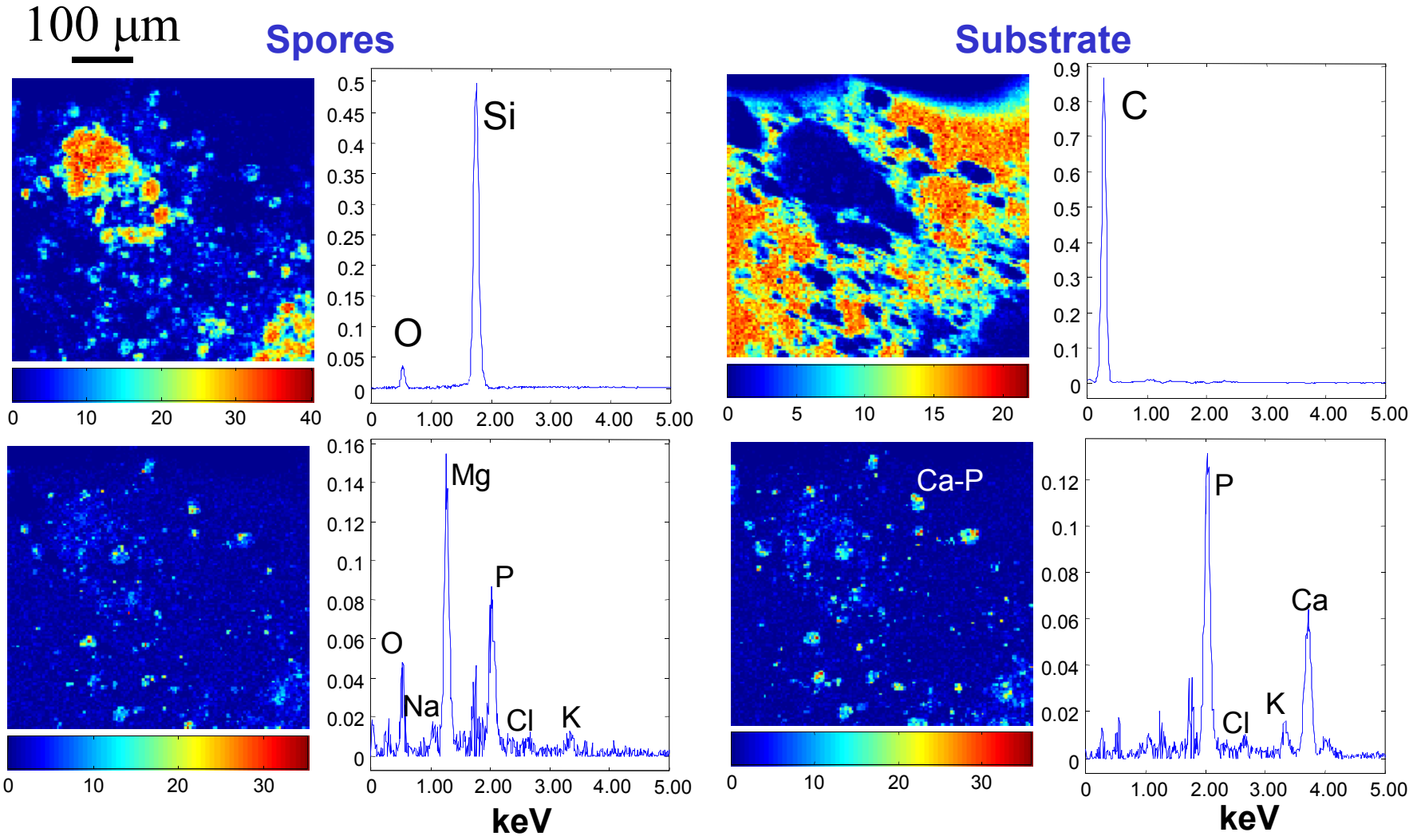


Secondary electron image of SiO nano-particles on *Bt* spores.



EDS acquired at 10 kV

SEM – Spectral images of weaponized surrogate material

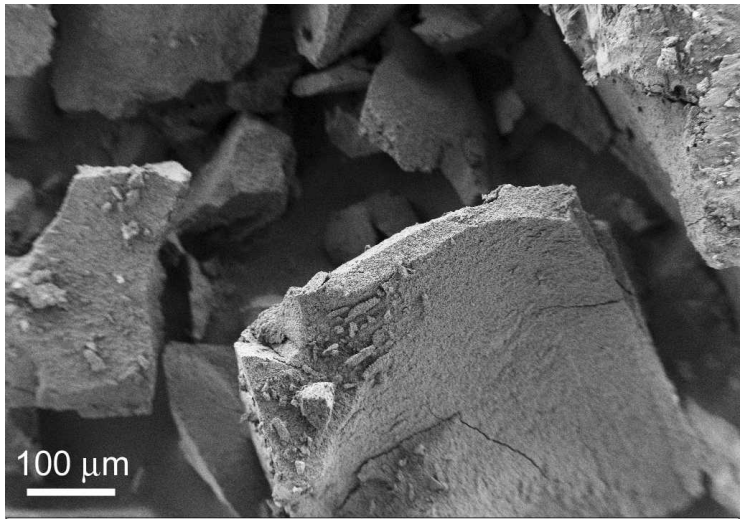


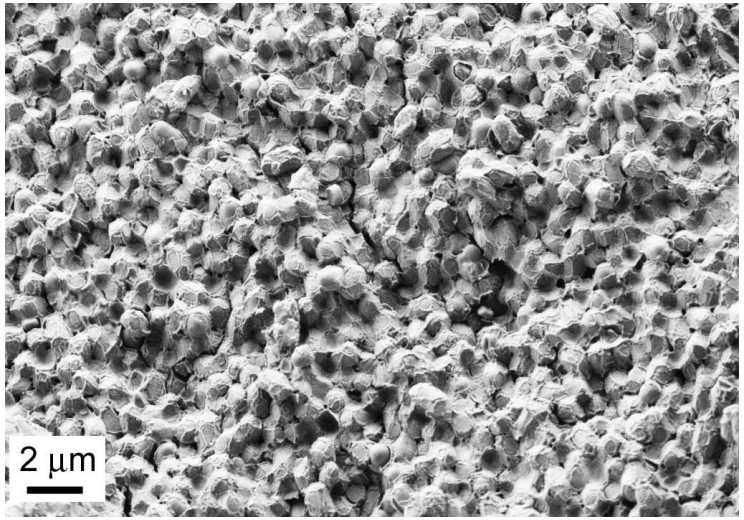
National Nuclear Security Administration

Sandia National Laboratories

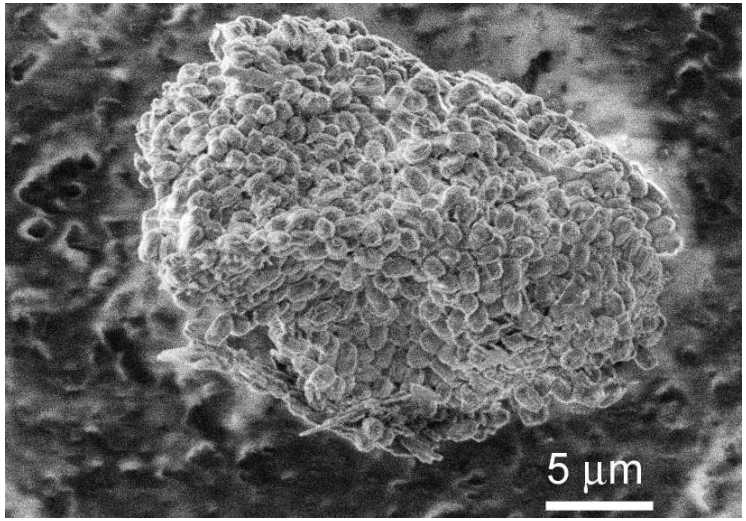
SEM of Leahy and New York Post Material

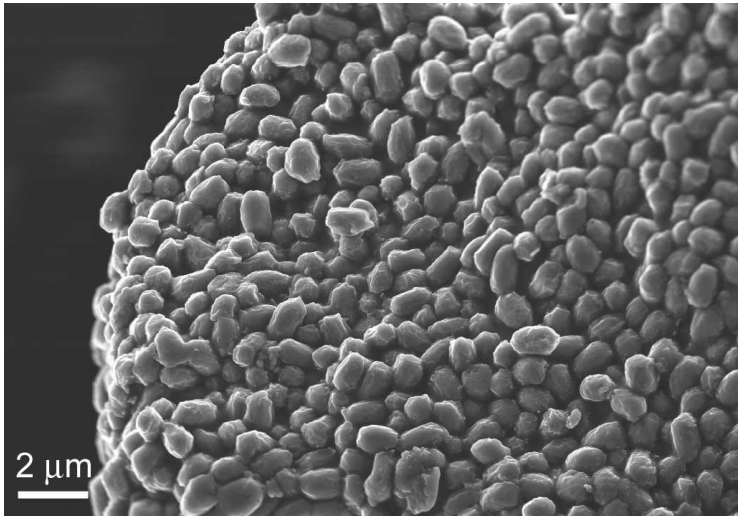
New York Post letter material

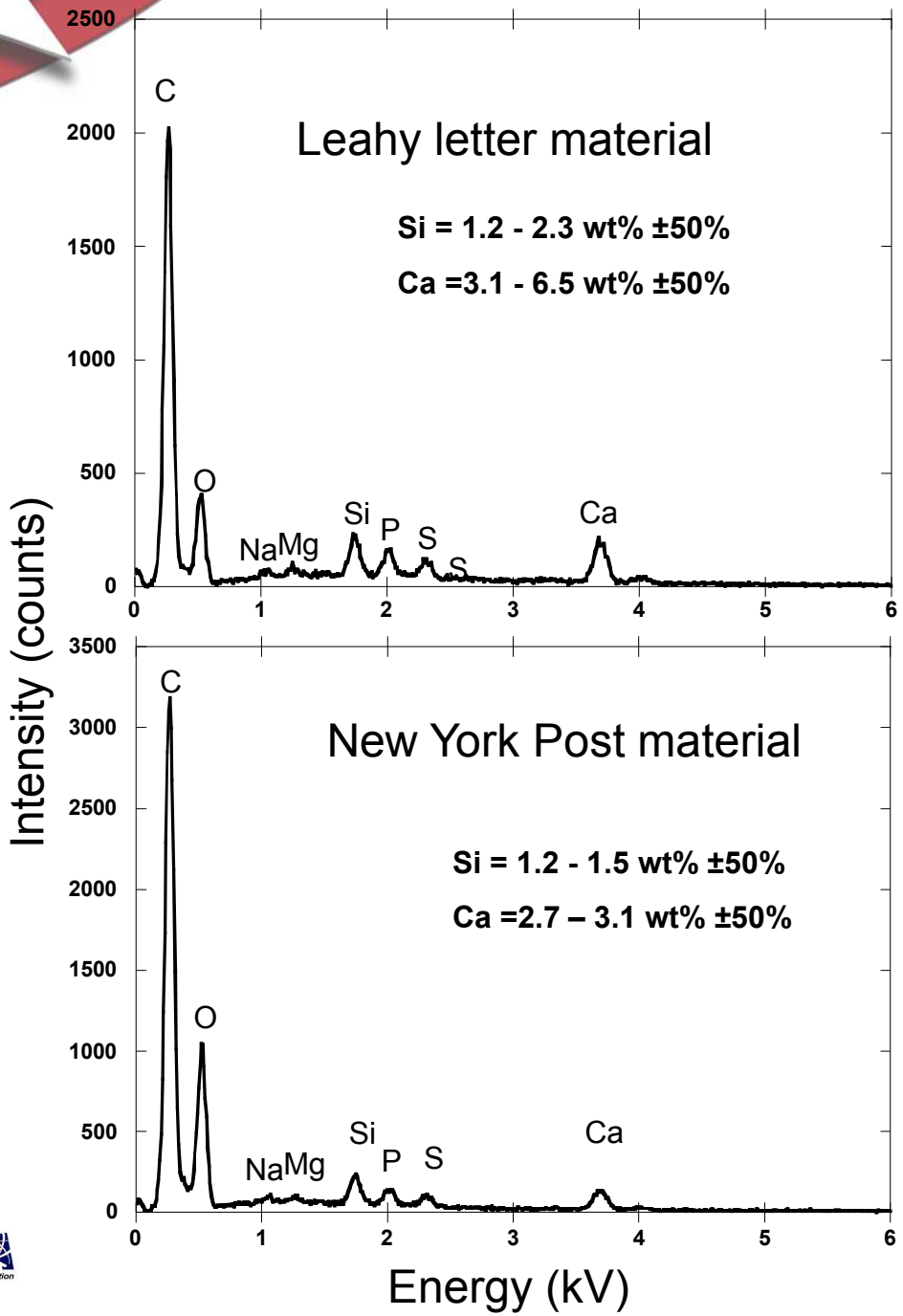


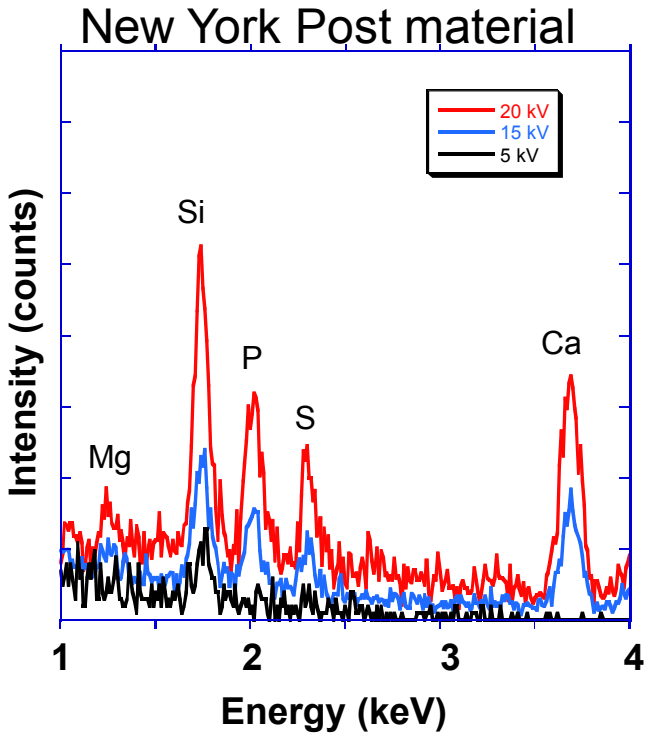


Leahy letter material









5 kV= 300 nm

15 kV = 2100 nm

20 kV= 3300 nm

Lower voltages produce more surface elemental information.

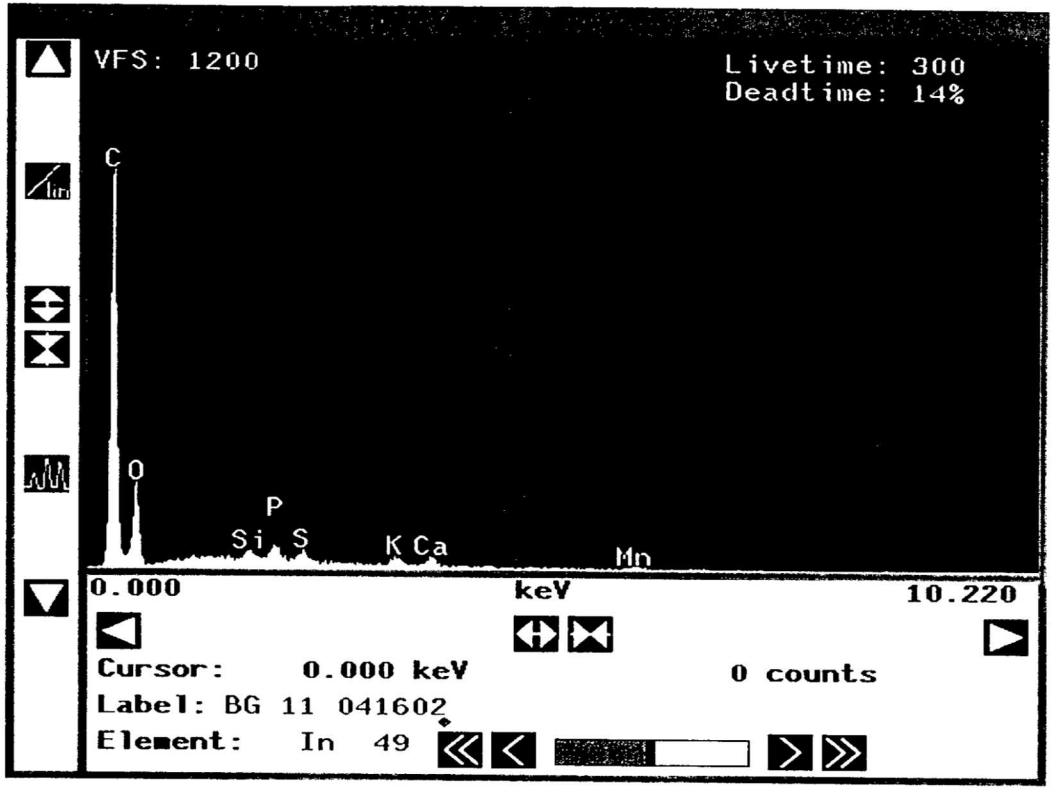
Very small amount of Si detected at 5 kV therefore Si is locate away from the spore surface.

National Nuclear Security Administration

Sandia National Laboratories

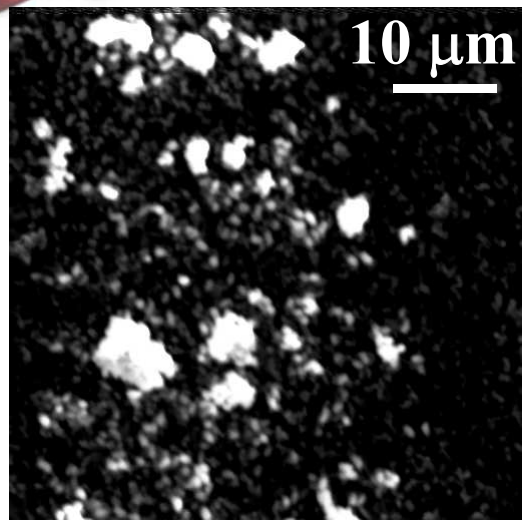
Bulk EDS Spectrum from Edgewood Report*

***Bacillus subtilis* var. *niger* spores grown in Casein Digest (CD) Medium (no indication that an anti-foam agent was added).**



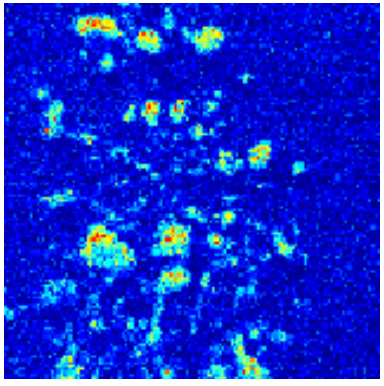
*L. F. Carey, D. C. St. Amant and M. A. Guelta, Production of *Bacillus* spores as a simulant for biological warfare agents, Edgewood Chemical Biological Center,ECBE-TR-372, April 2004.

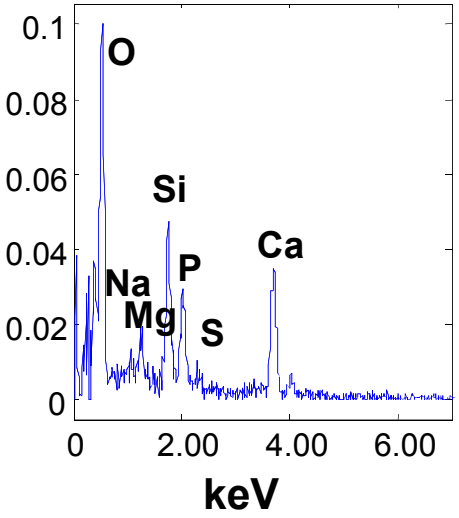
SEM – Spectral images of Leahy spore material



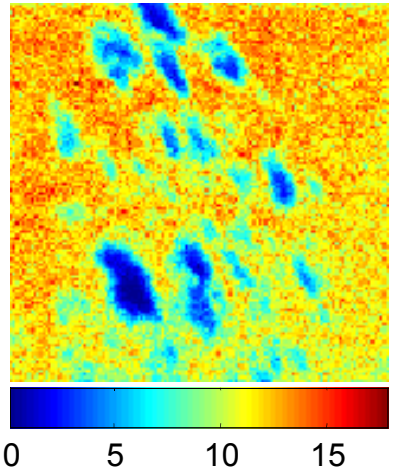
SEM Image of Leahy material

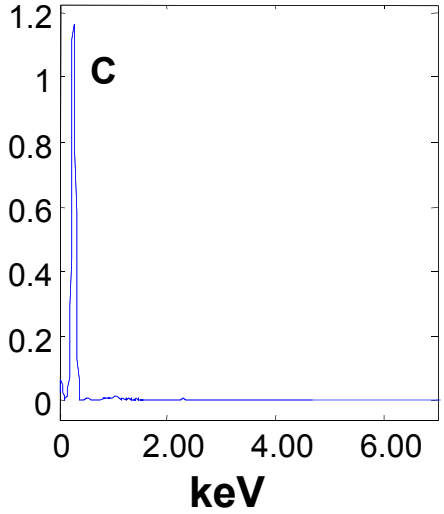
Spore material





Support material





Spectral Image components of Leahy material

NNSA National Nuclear Security Administration

Sandia National Laboratories

Summary of SEM Observations of spore materials

Microanalysis in the SEM shows that Si is present in the Leahy and New York Post materials.

But- microanalysis of bulk samples in the SEM lacks sufficient spatial resolution to show where the Si is located with respect to the spores.

Low kV shows Si is mostly on the interior of the spores.

Microanalysis in the SEM is can be made quantitative.

But not from samples like the powder attack materials.

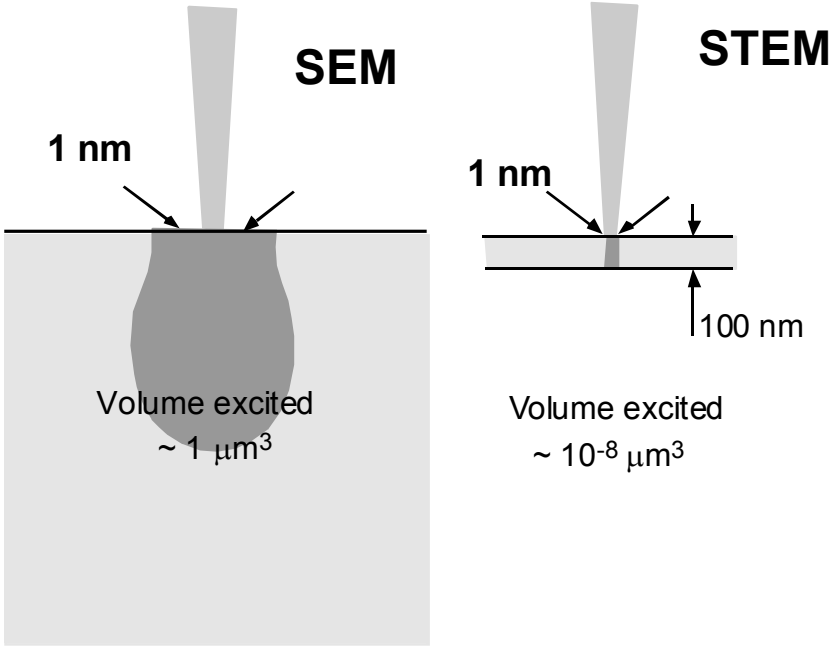
Spectral imaging with component analysis provides some useful information.

Comparison of SEM and STEM

SEM

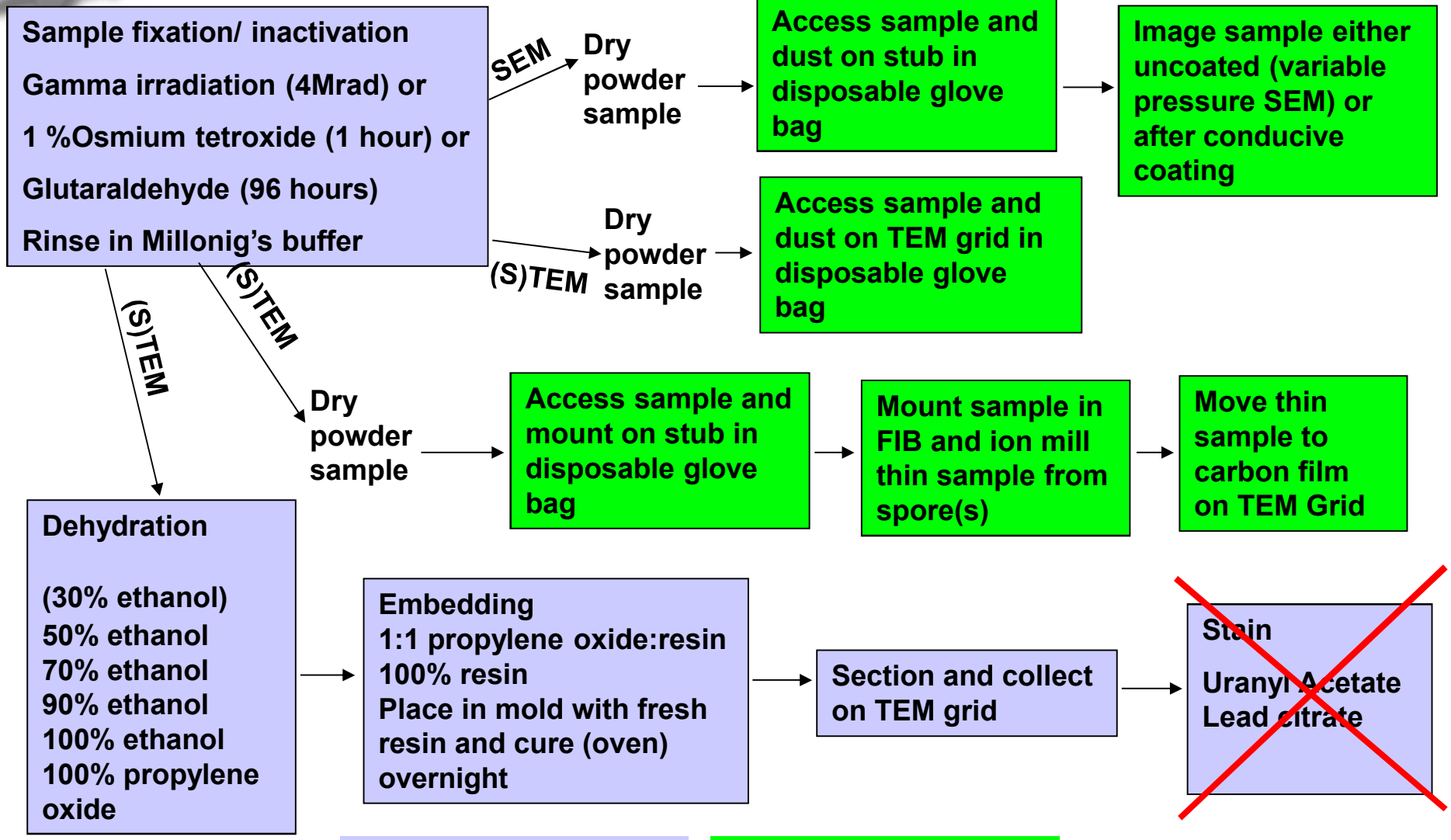
- Imaging – 0.6 nm currently
- Microanalysis – about 1 μm
- Elements – limited to >Be
- Diffraction for crystallography
- Instrumentation is expensive
- No sample preparation may be required

- **STEM**
- High Resolution Imaging – 0.2 nm
- Microanalysis – 1-2 nm spatial resolution
- Elements – limited to >Be
- Diffraction for crystallography
- Instrumentation is really expensive
- Electron transparent (thin) samples



In this study we make use of the characteristic x-rays generated by the electron/sample interactions.

Preparation of samples for STEM or SEM

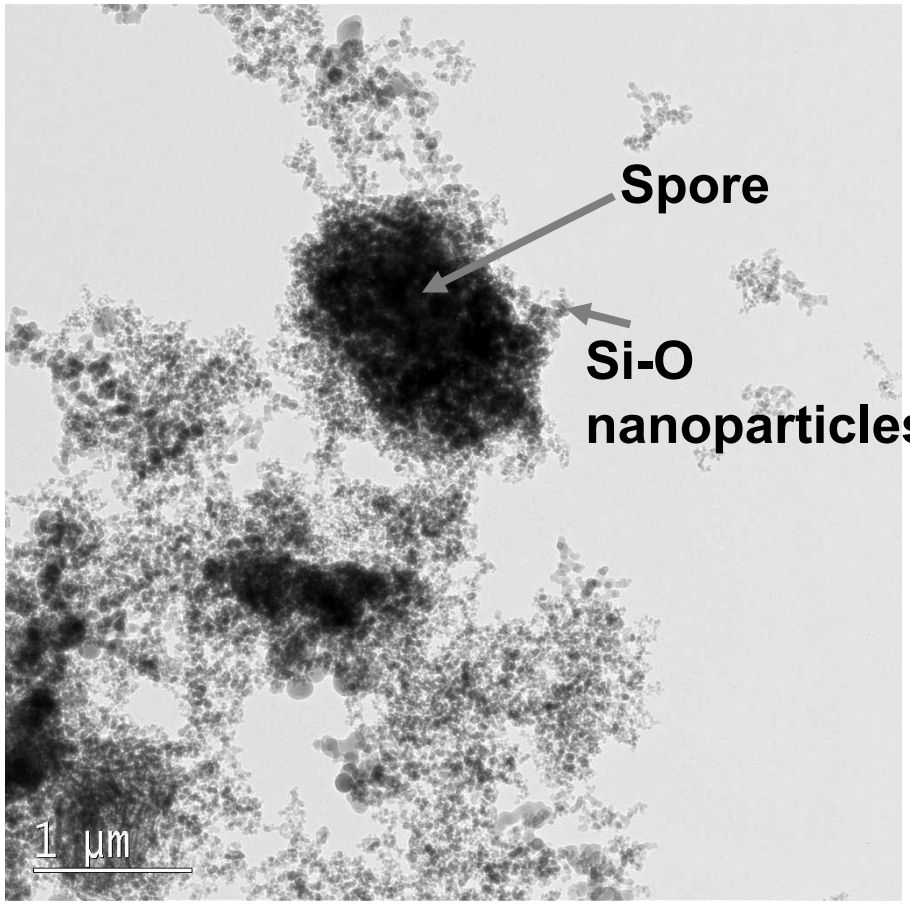


National Nuclear Security Administration

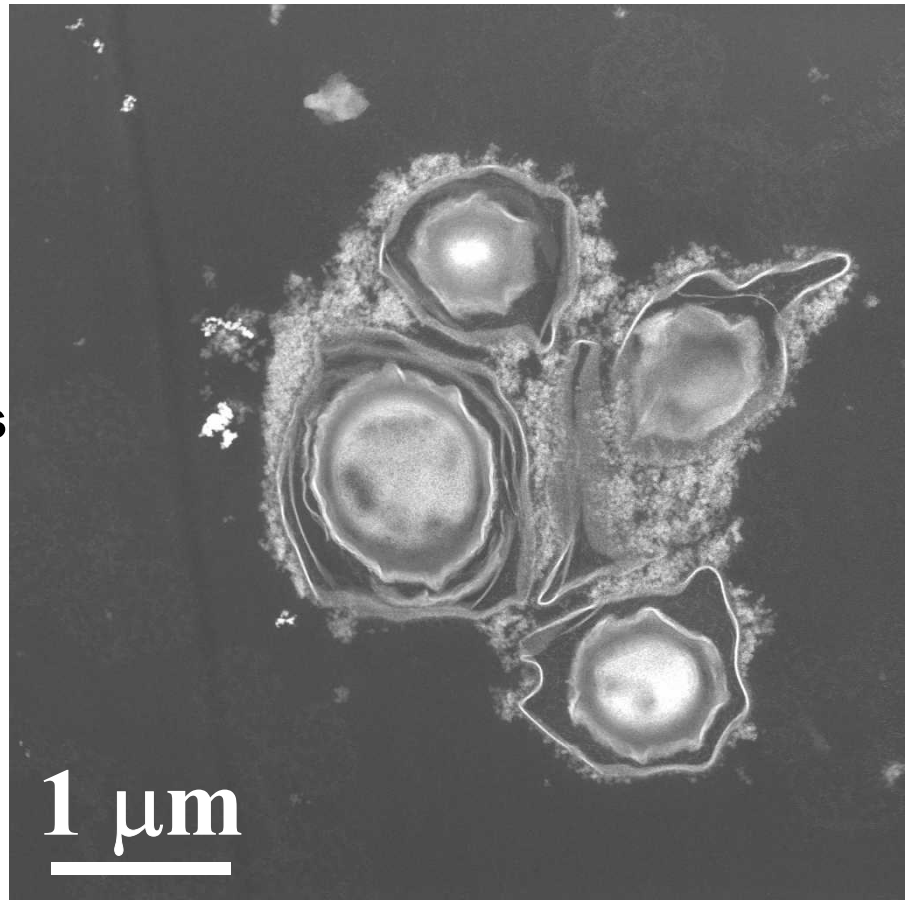
Sandia National Laboratories

Weaponized Bt Surrogate

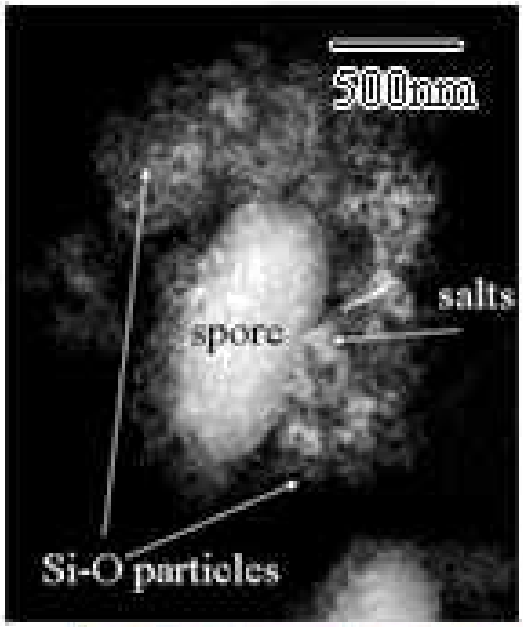
Bright Field TEM image



Annular Dark Field STEM image

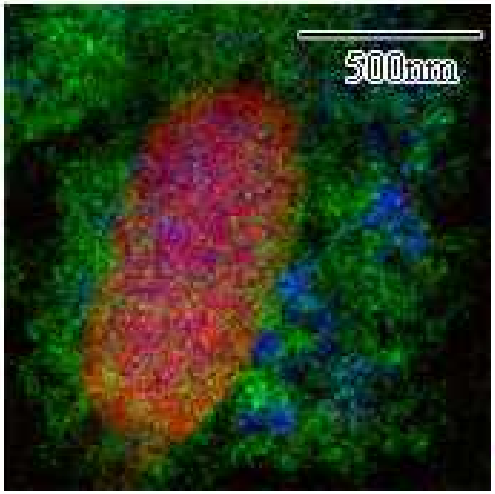


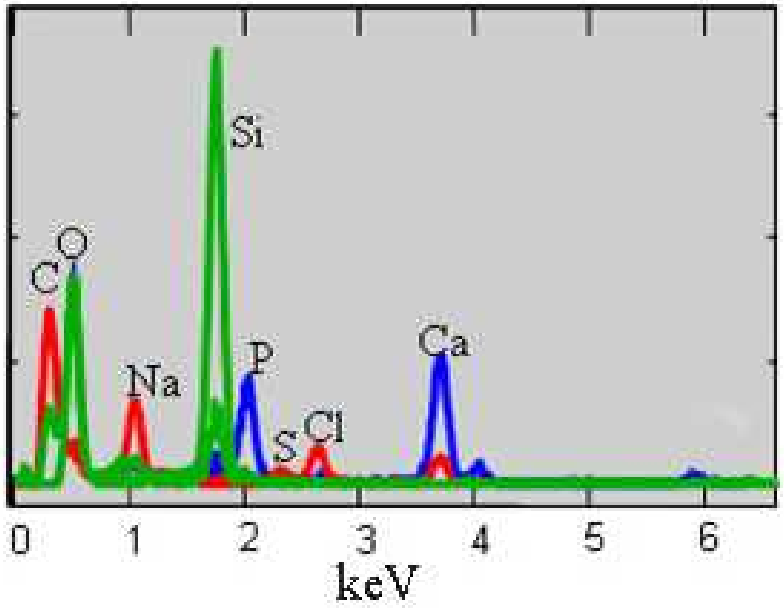
Spectrum imaging for elemental forensic signatures



- **Fluidized agent has silica nano-particles**
- **Ca-phosphate nano-particles present**
- **Na, Ca and Cl associated with spore body**

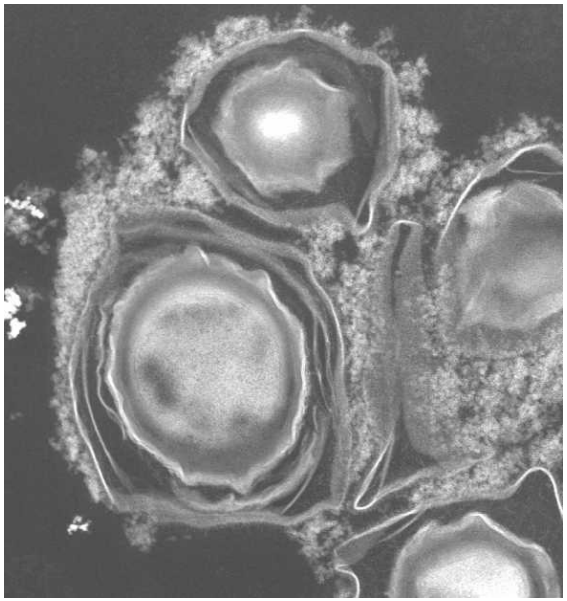
See: L. N. Brewer, J. A. Ohlhausen, P. G. Kotula and J. R. Michael, Forensic imaging of bioagents by X-ray and TOF-SIMS hyperspectral imaging", Forensic Science International, vol. 179, 2008, 98-106.

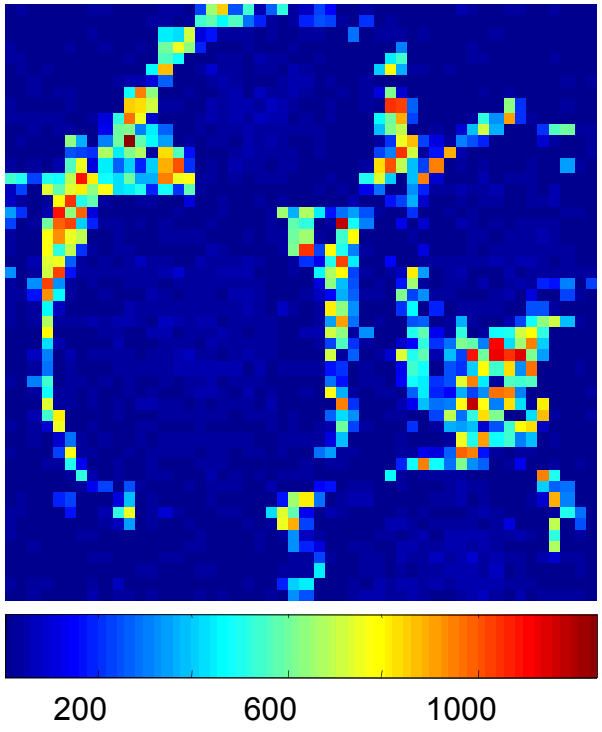


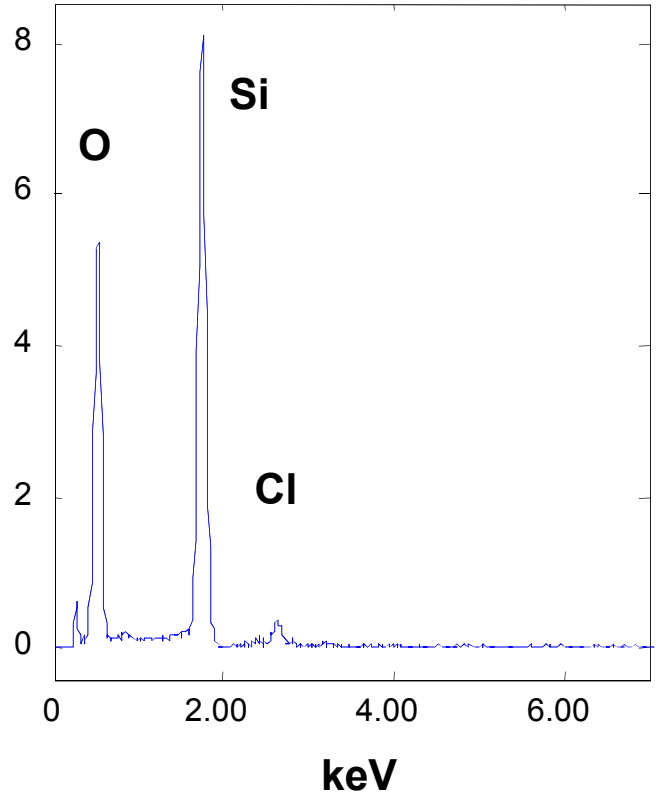


Weaponized Surrogate
Automated x-ray spectral image analysis

1 μm



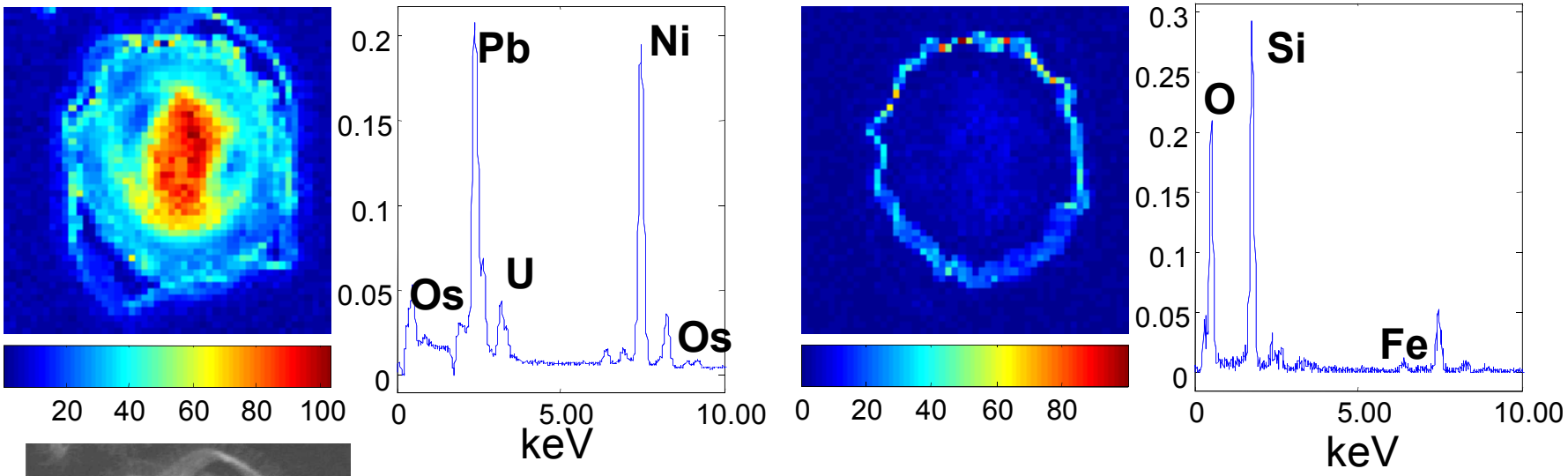


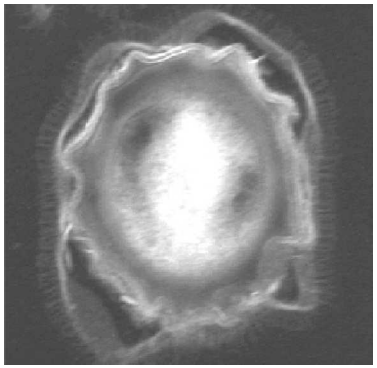


Si-O particles are found on the exosporium

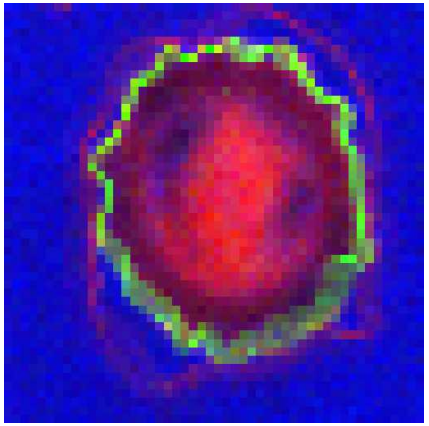
STEM microanalysis of Daschle letter material

Fixed, stained and ultramicrotomed section





STEM ADF



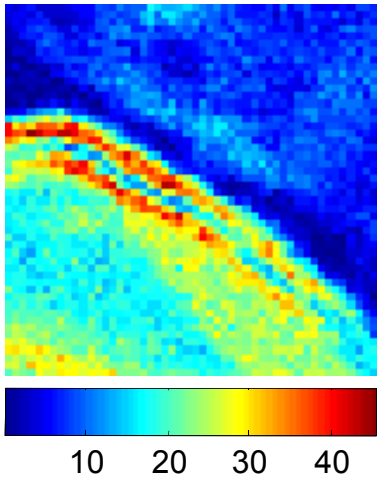
Si-O is on the spore coat and not the exosporium

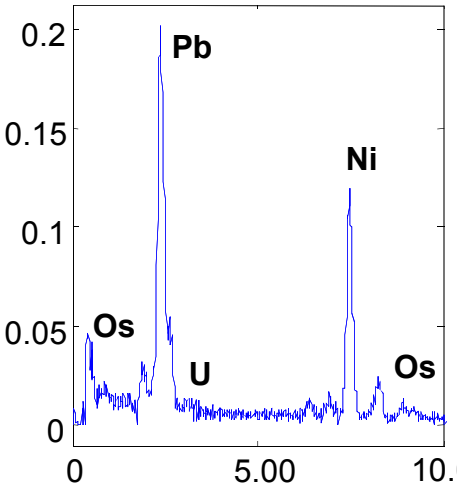
Elements from stain overlap other possible elemental signals

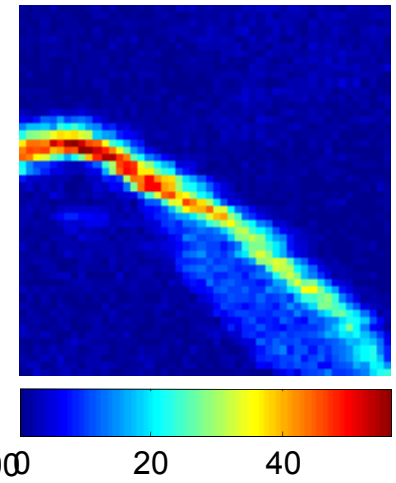
STEM microanalysis of Leahy letter material

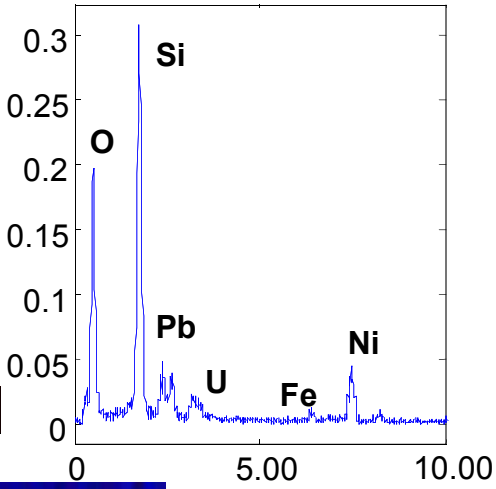
250 nm

Fixed,stained and ultramicrotomed section

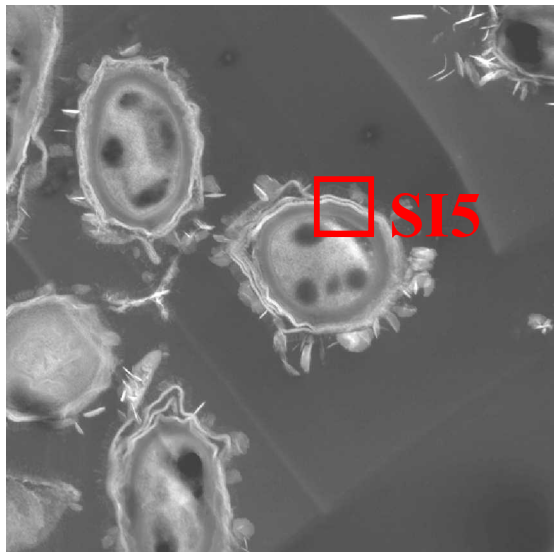


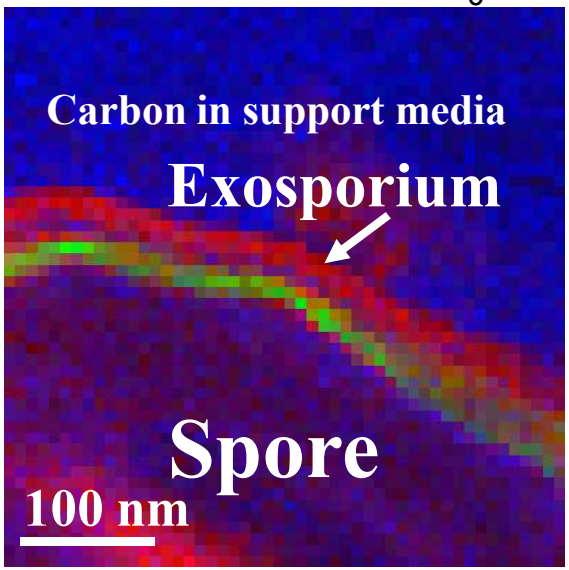






keV





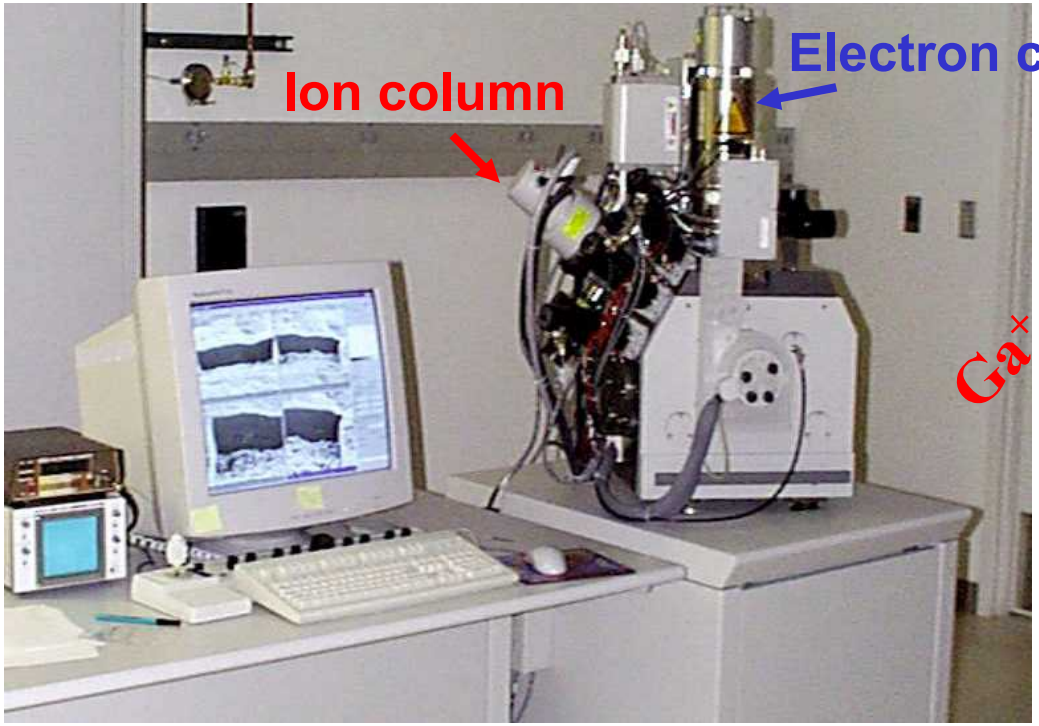
Red = Stain
Pb, U, C …
Green = Coating
Si, O…
Blue = Carbon in spore and support

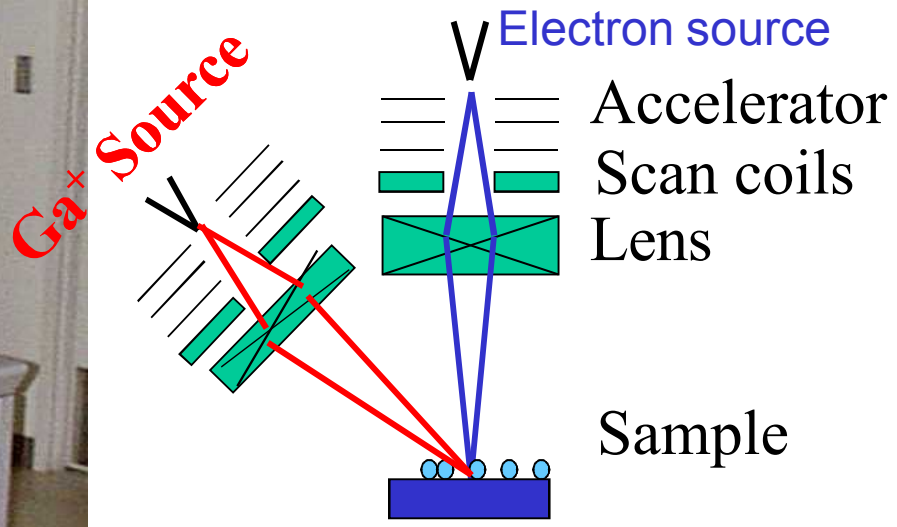
National Nuclear Security Administration

Sandia National Laboratories

Focused Ion-Beam (FIB) Tool/ Scanning Electron Microscope (SEM)

FIB/SEM

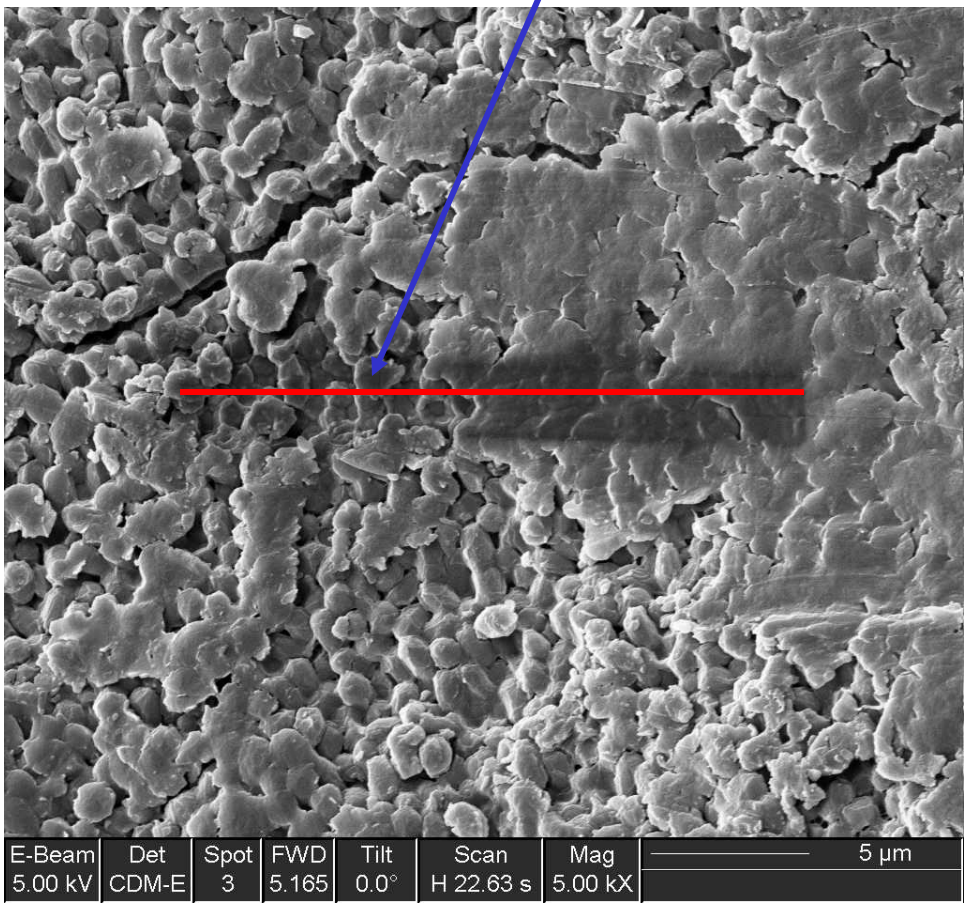




FIB allows unfixed/unstained site-specific TEM specimens to be prepared even of single spores

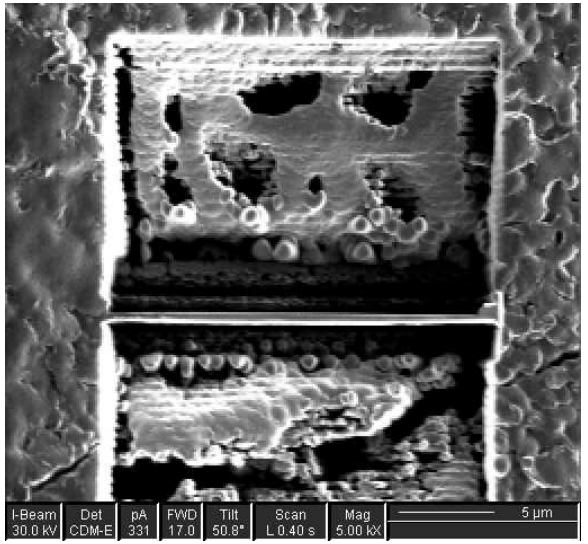
FIB Specimen Preparation

Region of TEM sample

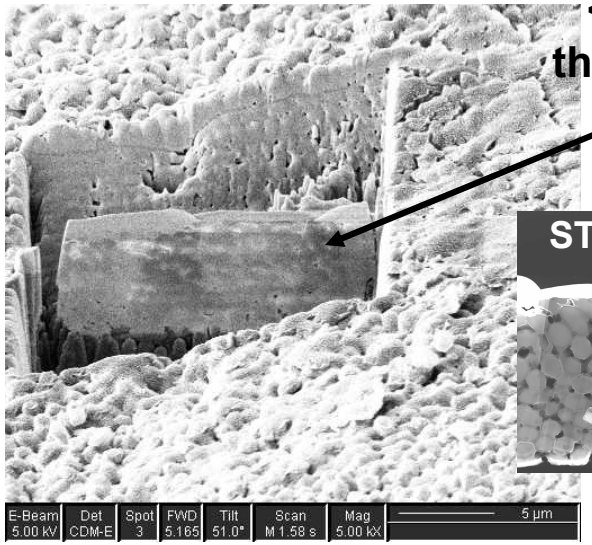


SEM of clump of spores.

Can also prepare specimens from isolated spores



Ion image of TEM specimen



SEM of TEM specimen ready to be extracted

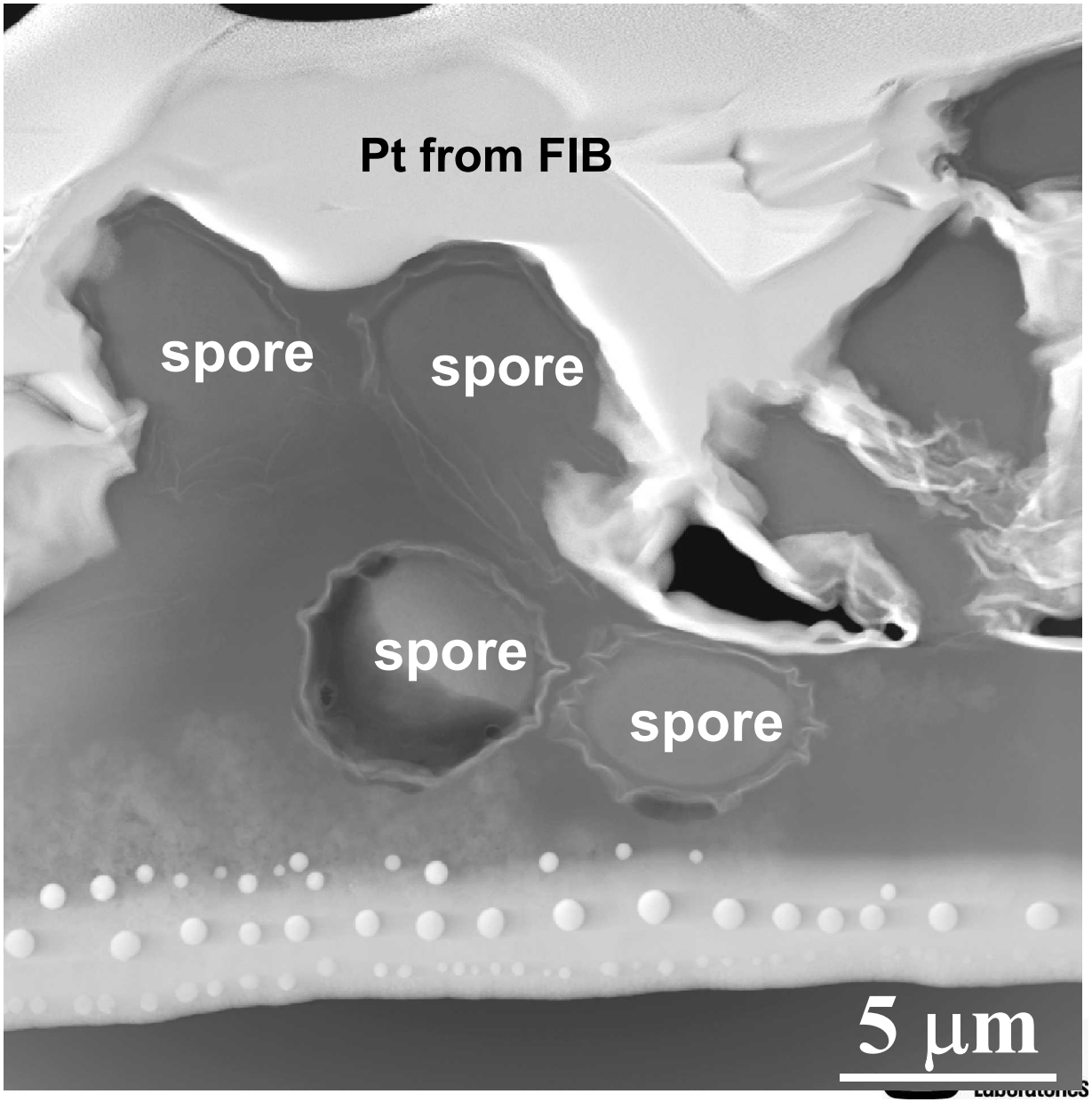
National Nuclear Security Administration

Sandia National Laboratories

Leahy Letter FIB Cross-section

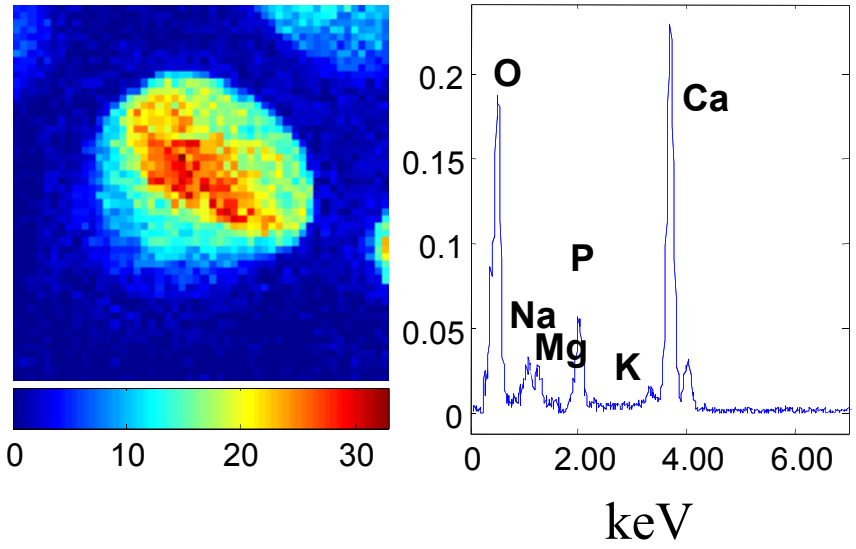
STEM Annular Dark-Field Image of spores in cross-section

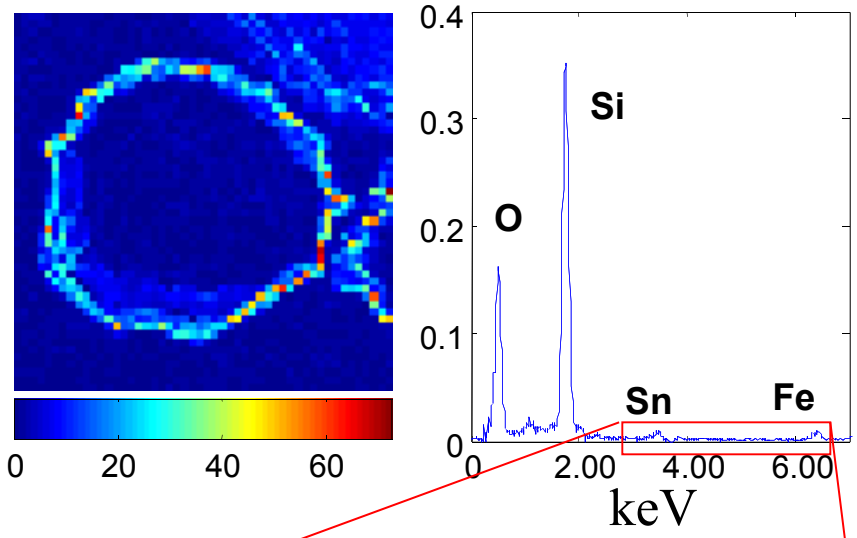
Cross-section sample made with FIB through irradiated, unfixed, unstained spores.

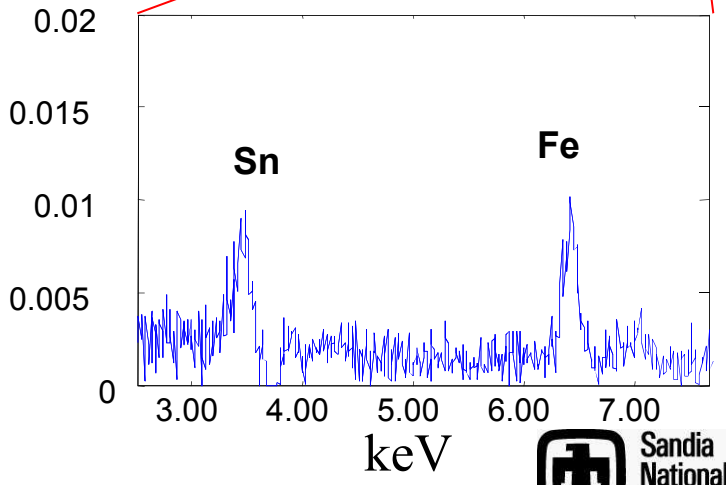


Leahy Letter FIB Cross-section – FIB prepared section

500 nm





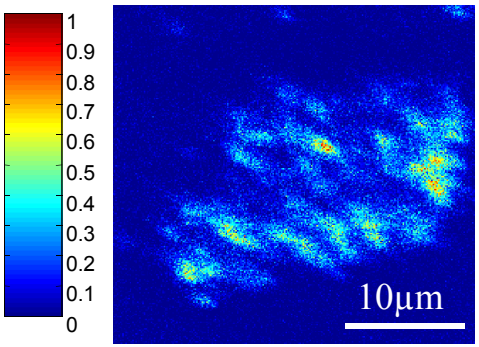


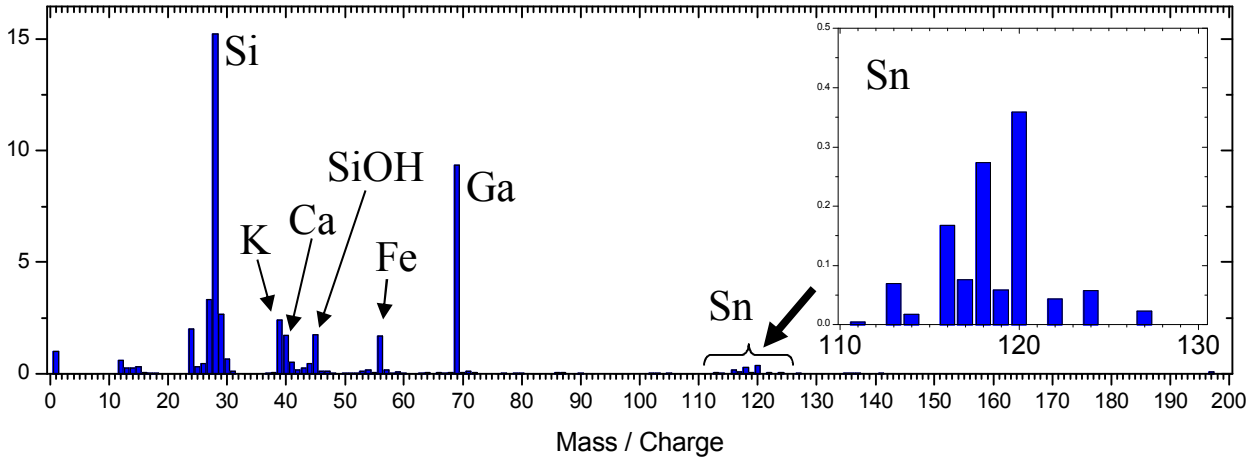
Additional chemistry, Sn, revealed in the absence of fixative and heavy metal stains

National Nuclear Security Administration

Sandia National Laboratories

Time-of-Flight Secondary Ion Mass Spectrometry (TOF-SIMS)

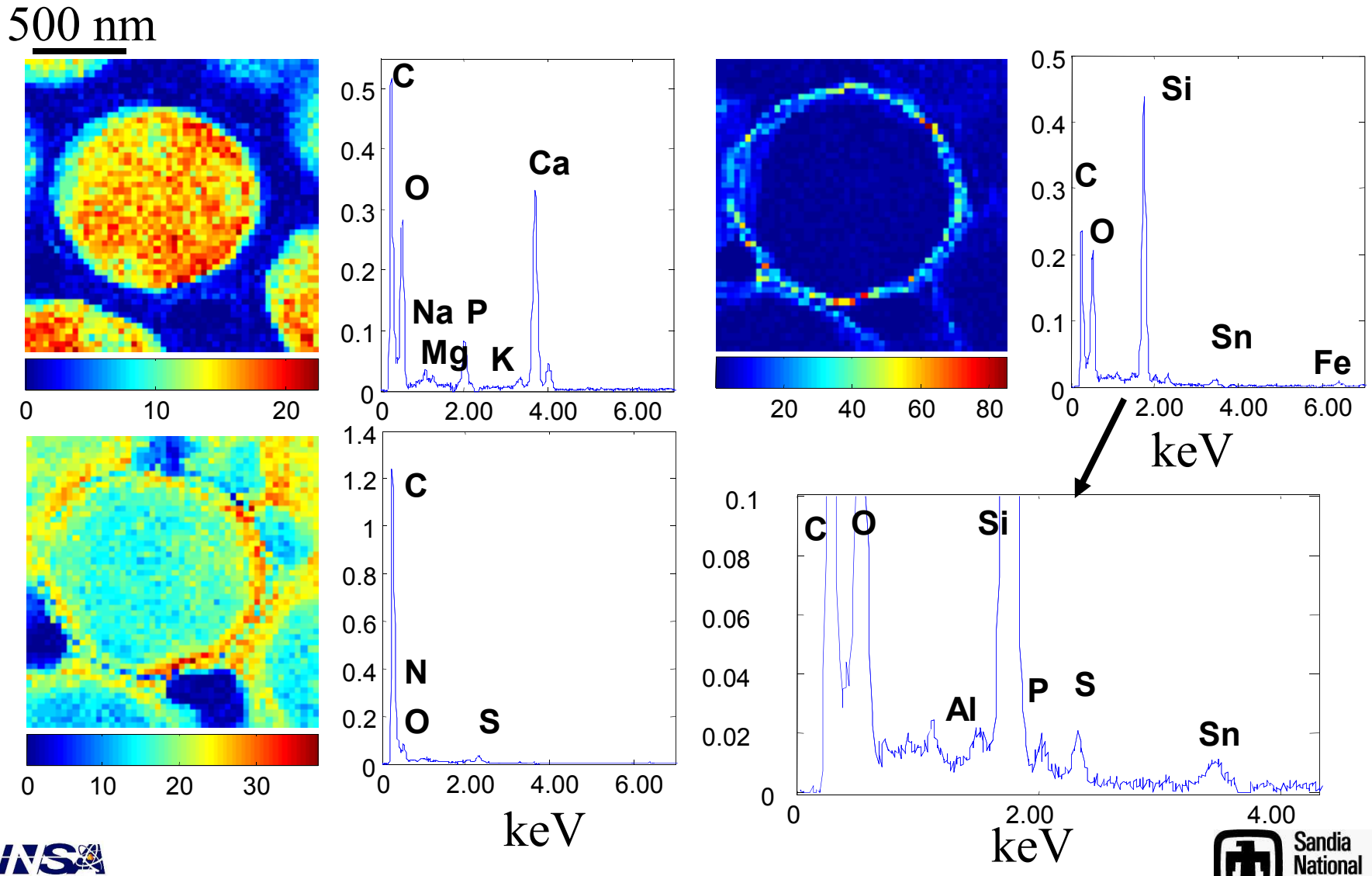




Ga^+ ion sputtering was used to remove surface of spore.

Layer that contains Si and O also has trace amounts of Sn and Fe

New York Post Material – FIB prepared section

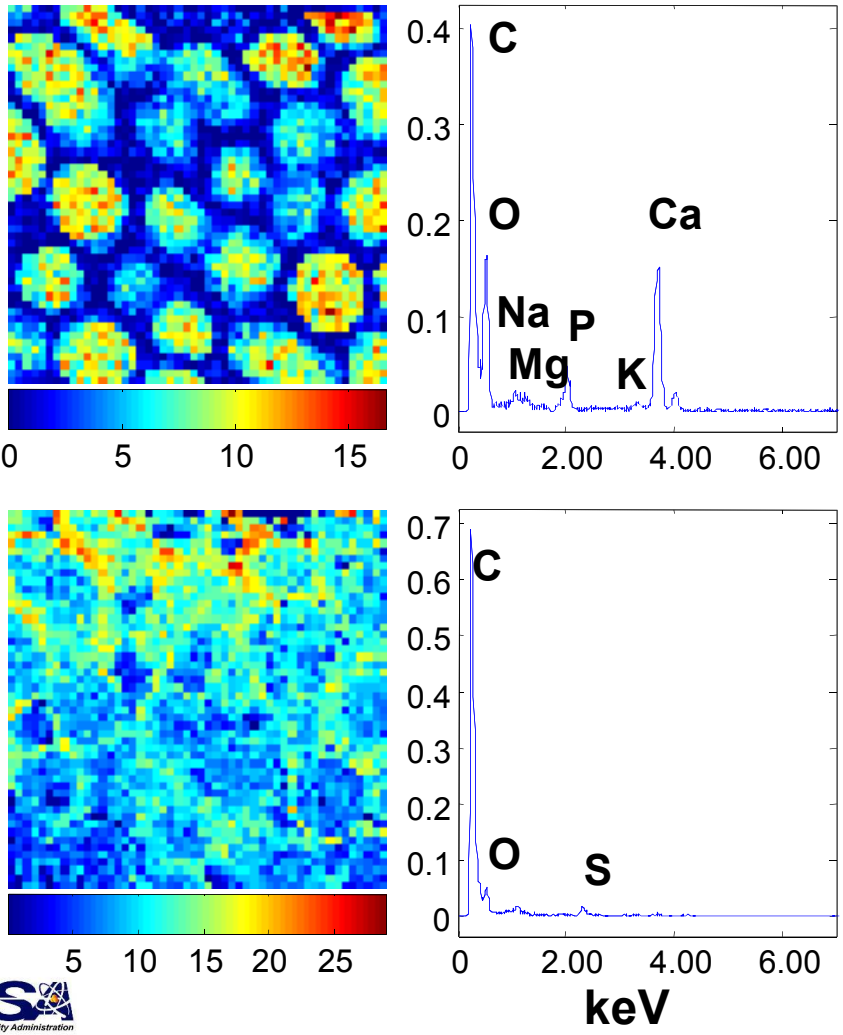


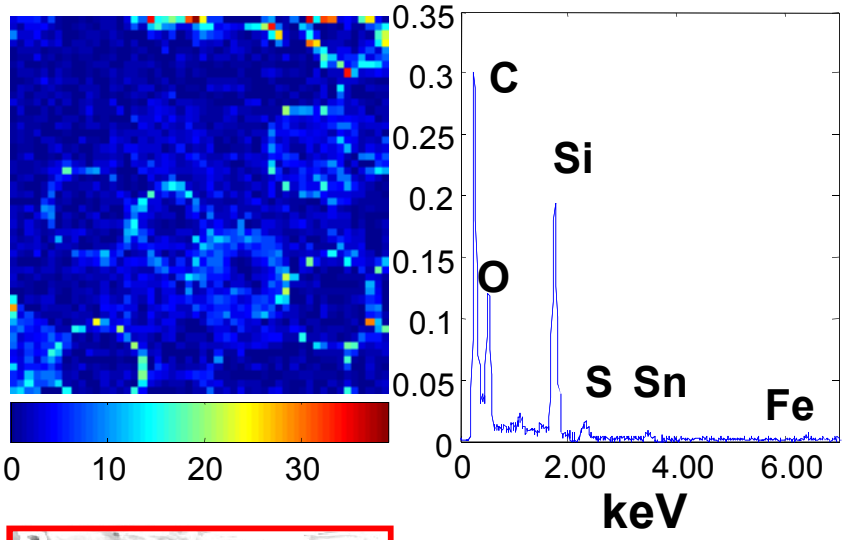
National Nuclear Security Administration

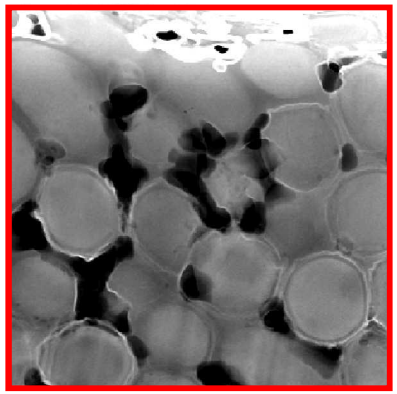
Sandia National Laboratories

New York Post Material – FIB prepared section

1 μm





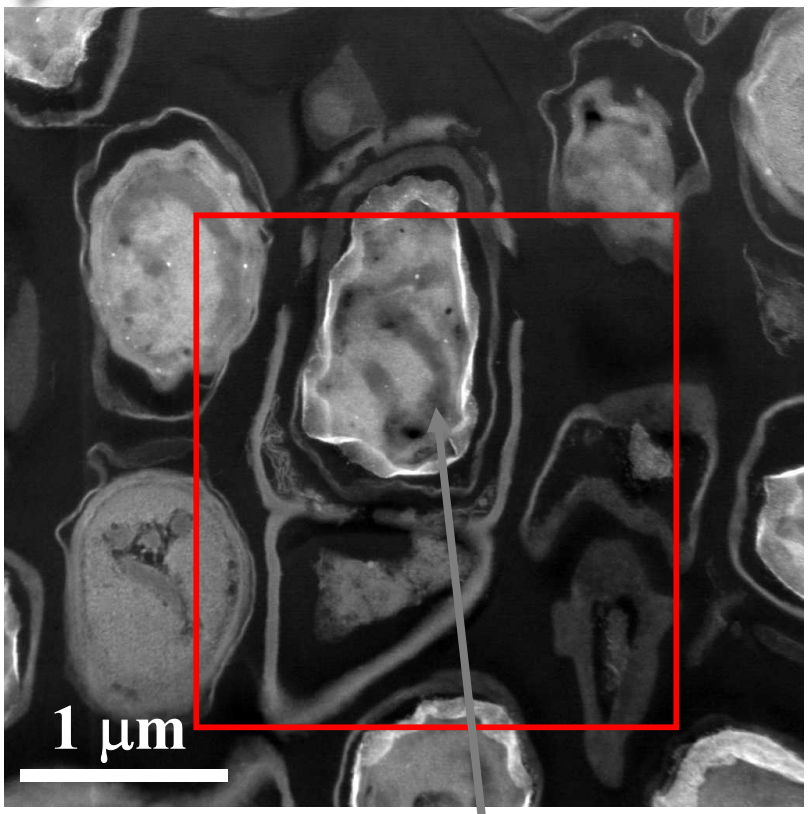


ADF STEM image

National Nuclear Security Administration

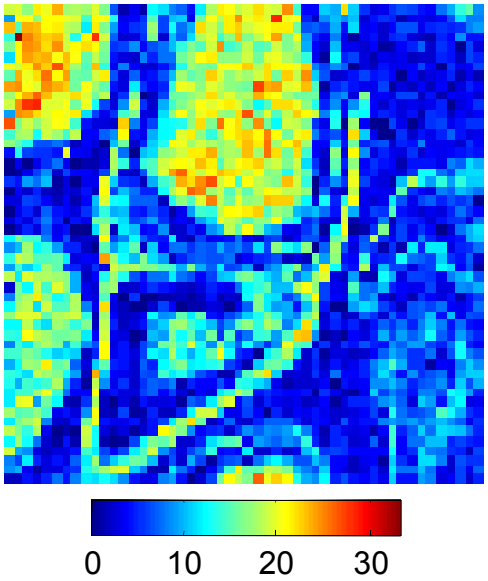
Sandia National Laboratories

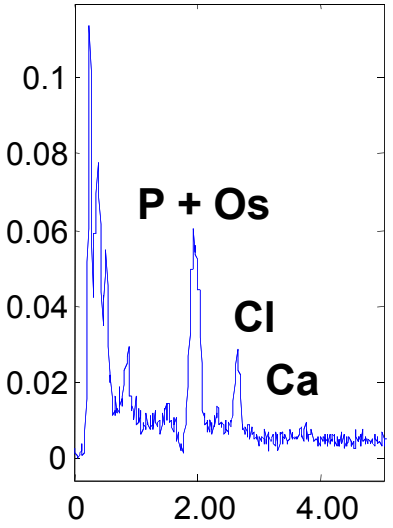
New York Post Microtomed, Unstained Section

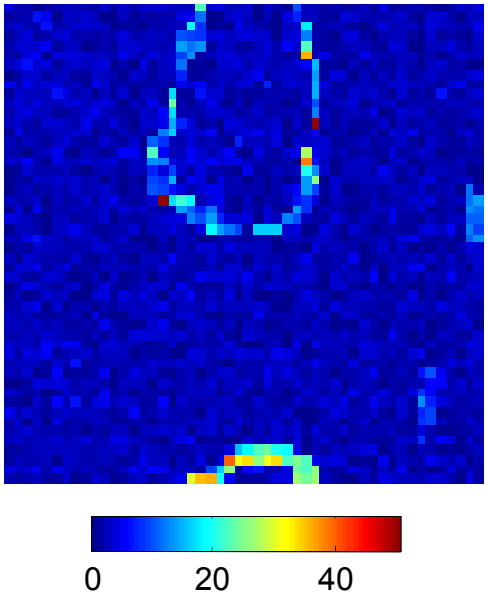


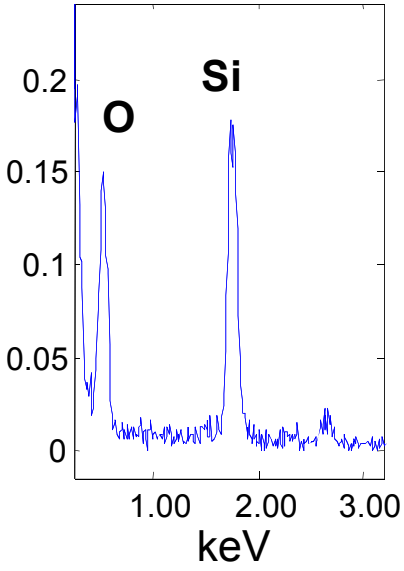
Vegetative cell with endospore.

Spore coat incorporates Si and O (Sn, Fe) within sporulating mother cell.







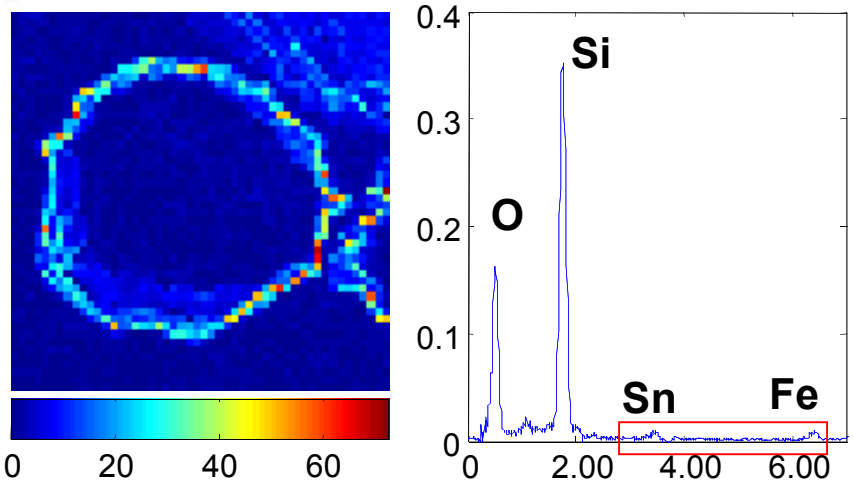


NNSA National Nuclear Security Administration

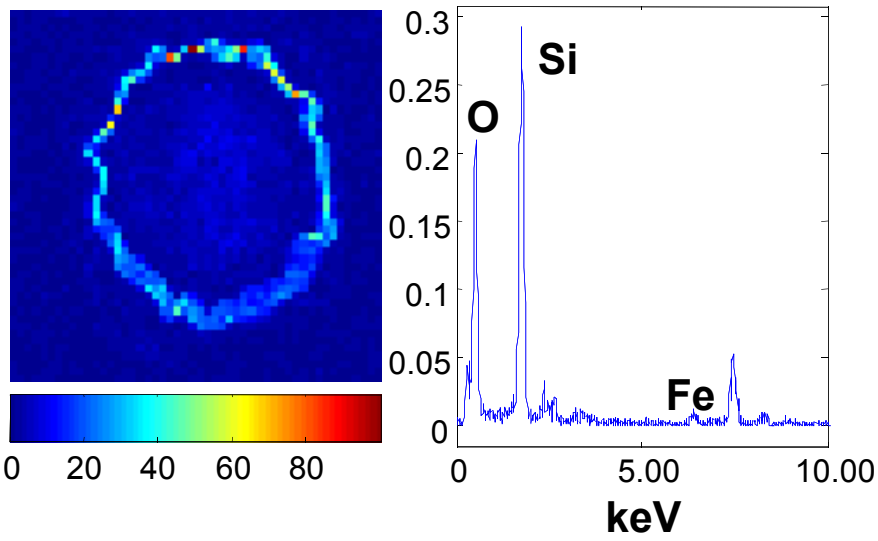
Sandia National Laboratories

Leahy, New York Post and Daschle are indistinguishable

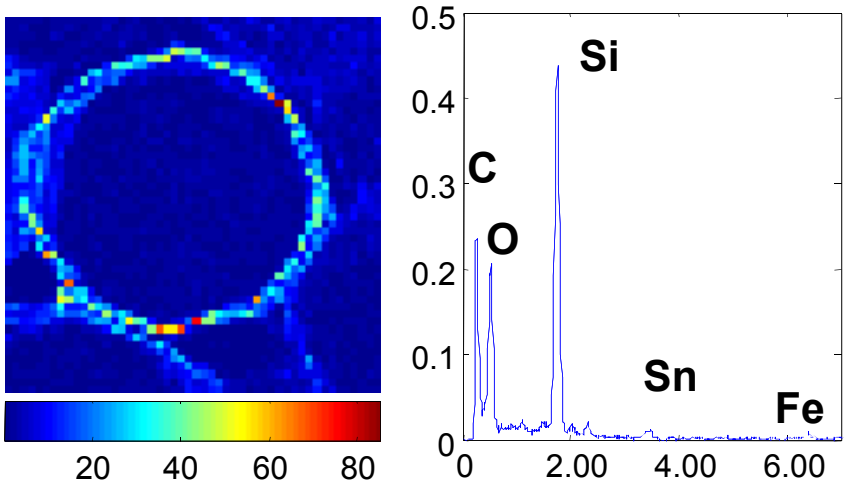
Leahy Material



Daschle Material



New York Post Material

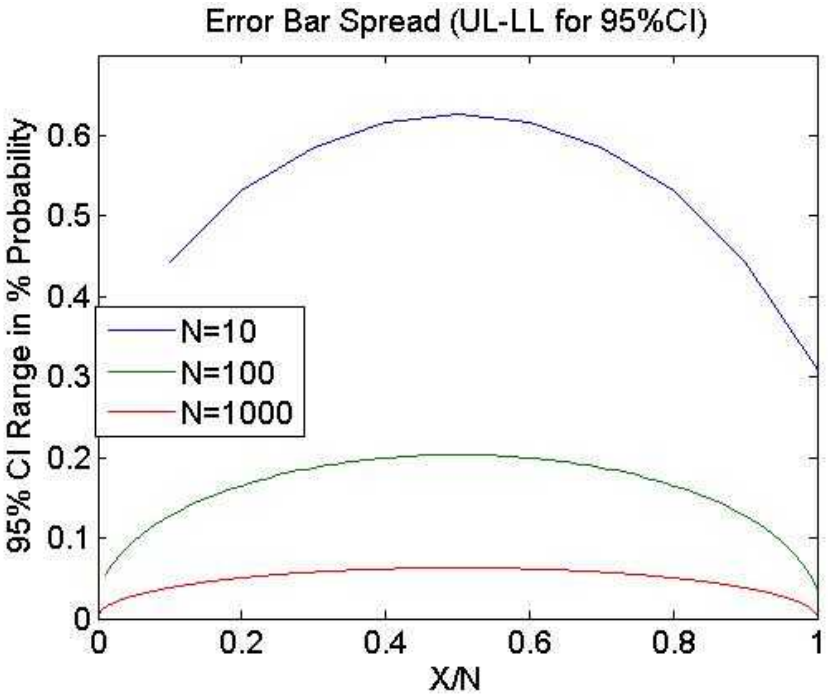


Spore coats on Leahy and New York Post samples are indistinguishable (both contain Si, Fe and Sn. Daschle appears the same (Si and Fe present, Sn is obscured by other elements in stain). Material from the Daschle letter was not made available for FIB sectioning.

National Nuclear Security Administration

Sandia National Laboratories

Importance of Spore Count



$$\frac{X \text{ Number of spores with a particular chemical feature}}{n \text{ Total number of spores analyzed}}$$

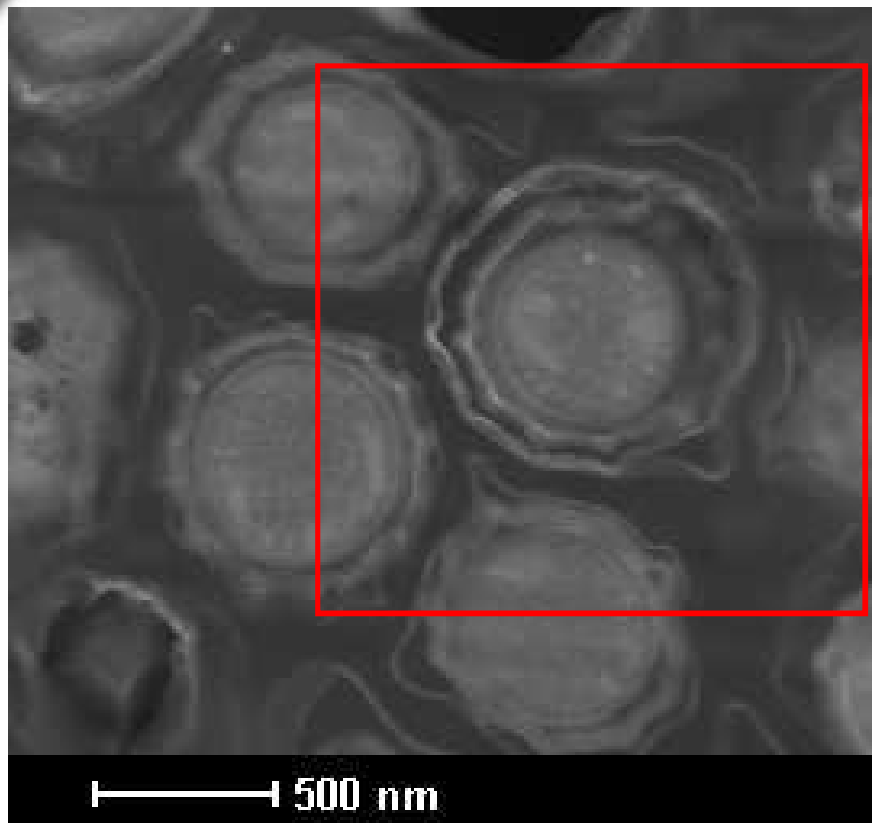
(Fraction of spores showing a certain chemical make-up)

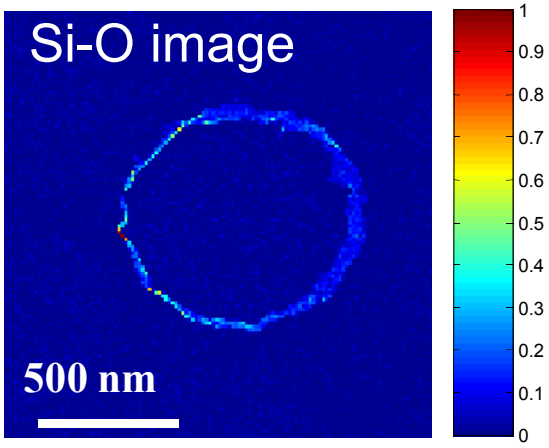
- **10 spores per sample is not sufficient for a reasonable comparison**
- **100 spores is both experimentally achievable and allow for reasonable comparison, but comparisons of spore count near 50% will lack real comparison power**
- **1000 spores allow precise comparisons but was experimentally unreasonable (~10 days and 10 TEM samples of analysis per bulk sample) until late development of new EDS detector for STEM in SEM.**

Analysis of fraction of spores with Si and O signature

Sample	# Analyzed	# with SiO	%	
SPS02.266	124	97	76	Leahy
SPS02.057	111	73	66	Daschle
SPS02.088	141	91	65	NYP
040255-1 level 2	163	42	26	
040255-1 level 5	161	17	11	
040255-1 level 8	172	50	29	
040030-2 level 2	94	6	6	RMR-1030
040030-2 level 5	118	0	0	RMR-1030
040030-2 level 8	113	7	6	RMR-1030
040089-1 level 2	98	0	0	RMR-1029
040089-1 level 5	115	0	0	RMR-1029
040089-1 level 8	91	0	0	RMR-1029

Sample 1030

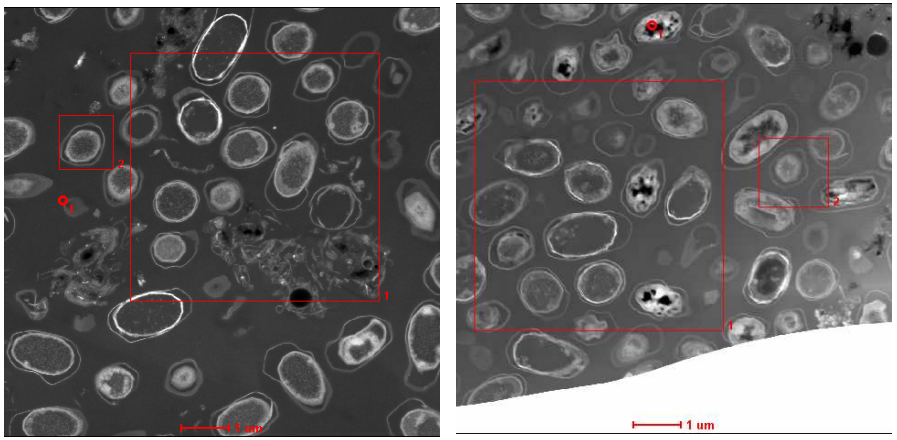


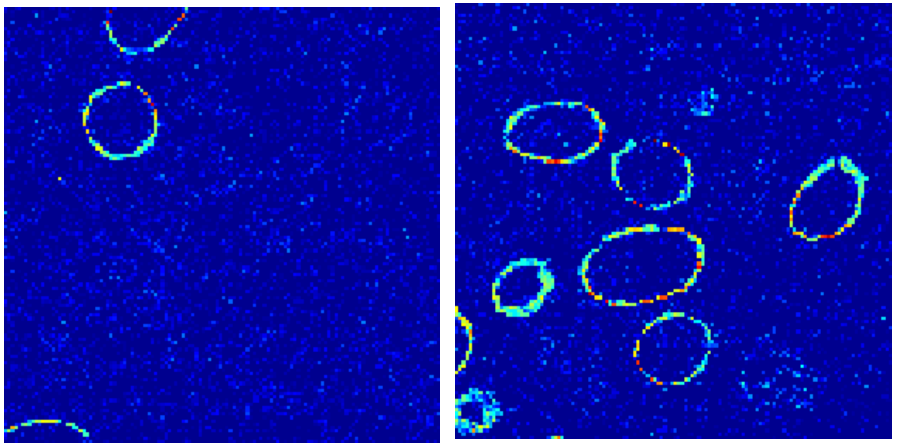


Bacillus anthracis Ames which was grown in shaker flasks at USAMRIID using Leighton-Doi media.

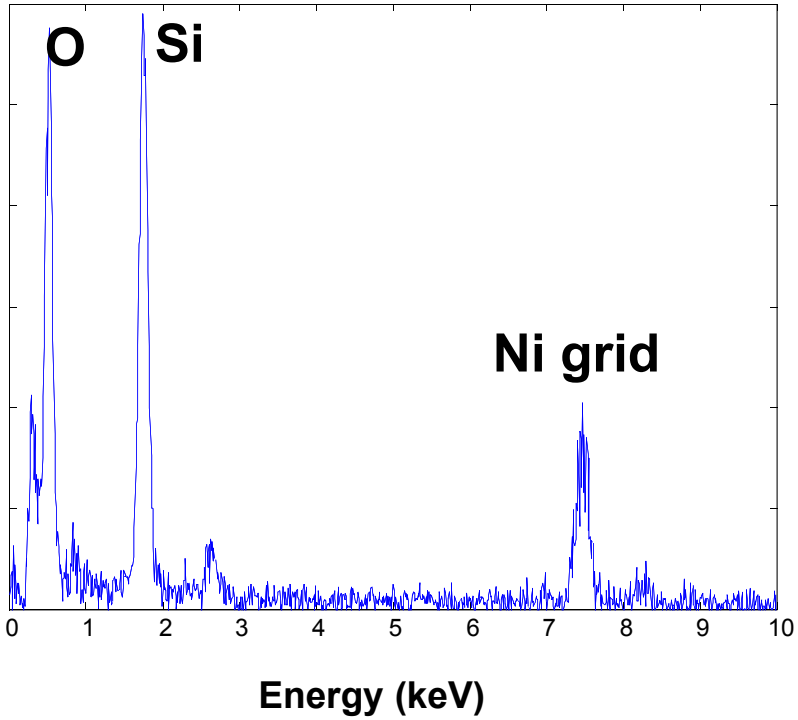
STEM images and Si and O component images of two samples of 040255

BA Ames grown via fermentation (Dugway) using Leighton-Doi media





Note variability in number of spores with Si and O elemental signature.



Si and O spectral component from spores

National Nuclear Security Administration

Sandia National Laboratories

Analysis of fraction of spores with Si and O signature

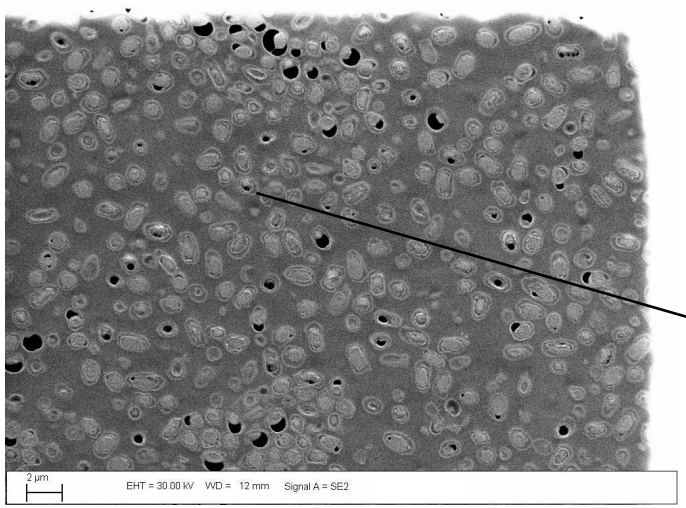
Sample	# Analyzed	# with SiO	%
NBFAC.071102.0001.0221.0002	1051	197	18.7
NBFAC.071102.0001.0228.0002	982	86	8.8
NBFAC.071102.0001.0232.0002	986	40	4.4
NBFAC.071102.0001.0230.0002	476	7	1.5
NBFAC.071102.0001.0235.0002	989	12	1.2

Sample was described as “evidence”, no further description given by FBI.

Analyzed using STEM in SEM technique

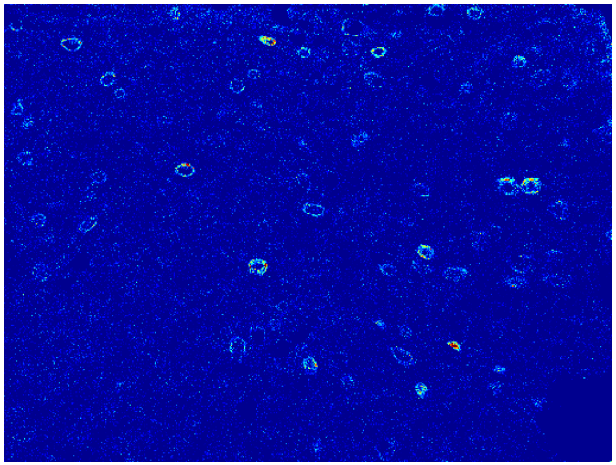
Si in the spore coat – new detector not sensitive to oxygen, STEM used to verify presence of oxygen

STEM in SEM of unstained, microtomed section

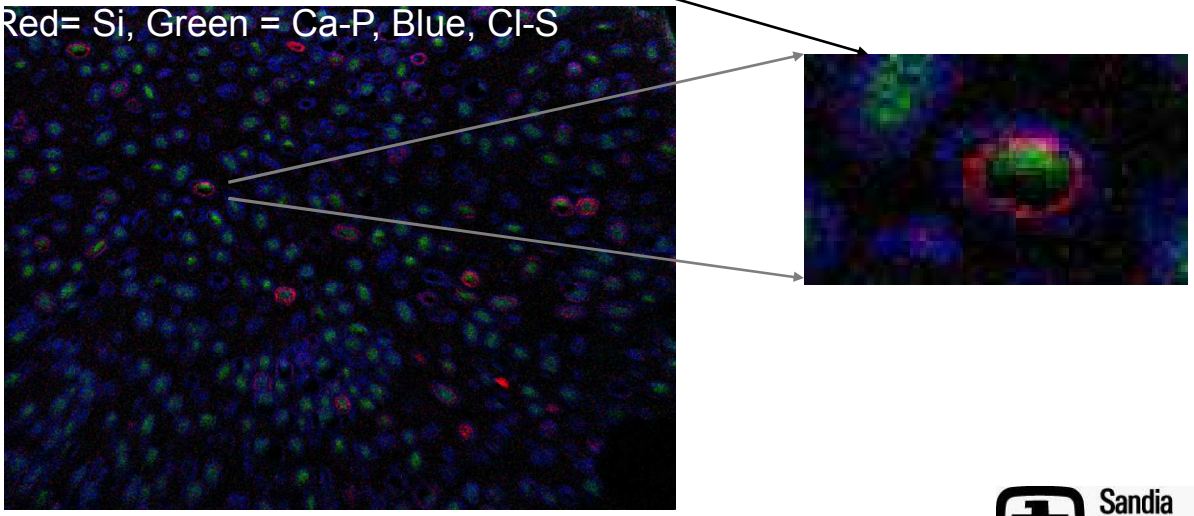


SEM defines field of view for spectral image acquisition

MSA identifies three chemical signatures



Si-containing spore coat



From this it is possible to count *x* and *n*

National Nuclear Security Administration

Sandia National Laboratories

Previous studies have shown Si on the coat*

Modifed CCY medium containing: $MgCl_2 \cdot 6H_2O$, $MnCl_2 \cdot 4H_2O$, $FeCl_3 \cdot 6H_2O$, $ZnCl_2$, $CaCl_2 \cdot 6H_2O$, KH_2PO_4, K_2HPO_4, glutamine, acid casein hydrolysate, enzymatic casein hydrolysate, enzymatic yeast extract and glycerol.

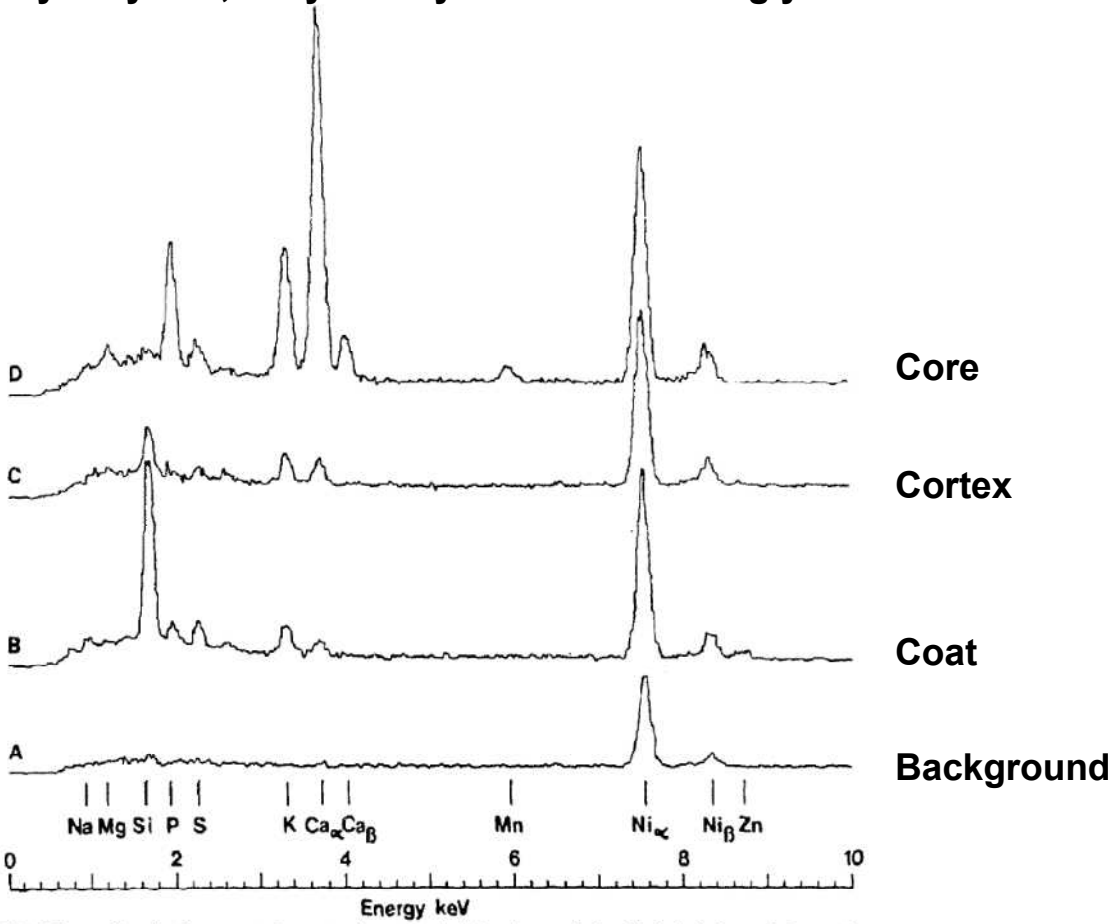


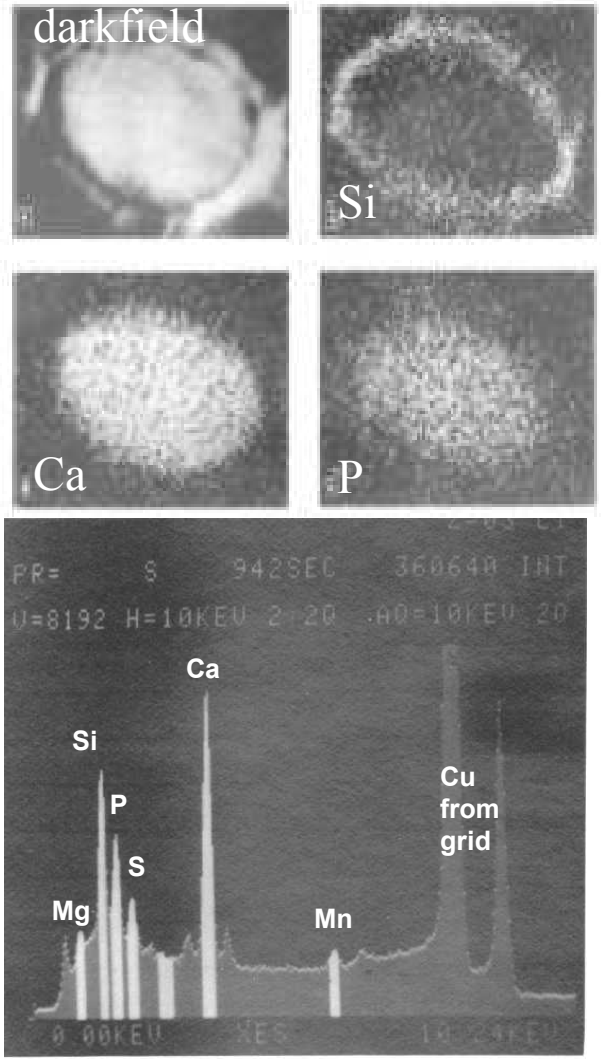
Fig. 3. Energy dispersive X-ray spectra for spot analyses at each of the points marked on Fig. 1. A, background; B, coat; C, cortex; D, core.

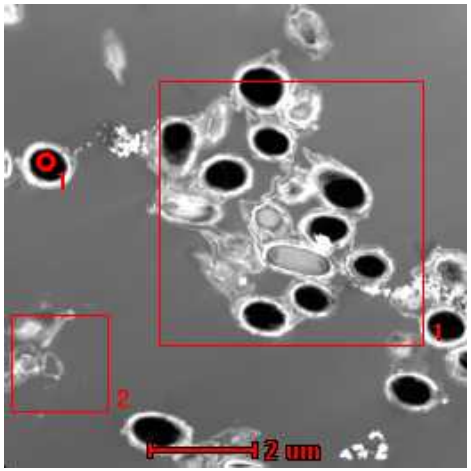
*Johnstone, K. et al., Location of metal ions on *Bacillus megaterium* spores by high-resolution electron probe x-ray microanalysis, FEMS microbiology Letters, vol. 7, 1980, p 97-101.

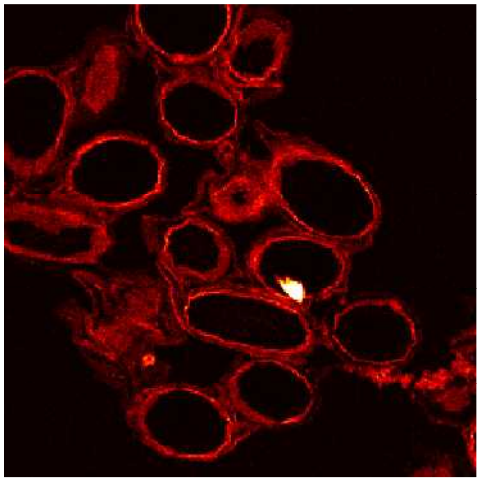
Analysis of samples from a previous study

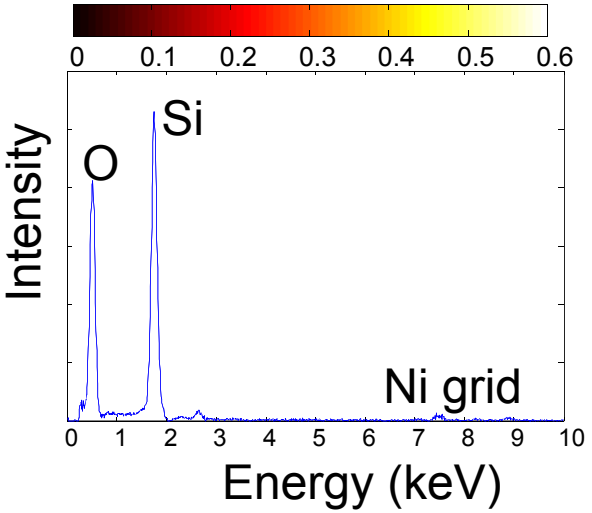
Grown in modified liquid G media, no anti-foam used

Bacillus cereus









Author's noted: "considerable variation in Si content both within and between different spore preparations,… unlikely to be due entirely to contamination."

M. Stewart et al., Journ. Bact., July 1980, p. 481-491

National Nuclear Security Administration

Sandia National Laboratories

Conclusions

- The NYP, Leahy and Daschle materials are indistinguishable elementally at the spore level.
- NYP, Leahy and Daschle materials all have similar fraction of spores with Si-O in spore coat
- Si-O signature found on endospores in New York Post sample
- The letter powders are not unique with respect to Si and O elemental signatures. Examples from the literature and from samples grown for this study.
- SEM and STEM have proven useful for spore characterization