Characterization of Bacterial Spores by NanoSIMS

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Anthrax attacks highlighted the needs for new bioforensics methods

- Genetics alone cannot answer critical attribution questions:
  - How was the agent prepared?
  - What materials were used?

Our Objectives:

- Develop protocols for analyzing individual bio-weapon particles
- Build a library of elemental and isotopic signatures
- Relate signatures to process and time of manufacture
- Combine signatures to assist attribution efforts
Bioweapons contain a wealth of signatures

- ~1 micron spore size
- BW agents are heterogeneous:
  - Spores zoned at nanometer scale
  - Weaponizing additives
  - BW products include environmental trace evidence
- Complex materials containing multiple chemical & isotope signatures
- The challenge is extracting meaningful signatures from complex materials
NanoSIMS is a state-of-the-art forensics tool

- High resolution & sensitivity
- Trace element and isotopic characterization at sub micron scale
- 1 of 18 worldwide

Applications
- Nuclear forensics
- Bioforensics

LLNL NanoSIMS Laboratory
We are developing NanoSIMS SOPs

- Initiated by LLNL Forensic Science Center for nuclear forensics
  —ISO certification in process
- Supported by DHS NBFAC for bioforensics
A surface sputtering technique

- Primary beam scans sample surface to produce secondary ions
- Secondary ions detected to produce quantitative digital images
- Simultaneous detection of 5 species
- High sensitivity: $\to 5\%$ useful yield

NanoSIMS 50 schematic

To mass spectrometer

Secondary ions

Secondary ions

Primary ions scanned over sample

Secondary ions detected to produce quantitative ion images

Sputtering collision cascade

Primary beam scans sample surface to produce secondary ions

Secondary ions detected to produce quantitative digital images

Simultaneous detection of 5 species

High sensitivity: $\to 5\%$ useful yield

50 nm beam diameter under ideal conditions

0.5 m
Spatial Resolution = Beam Diameter

- Standard dynamic SIMS: Min. $\Phi \sim 1 \mu m$
- ToF-SIMS: Min. $\Phi \sim 250 \text{ nm}$
- NanoSIMS: Min. $\Phi \sim 50 \text{ nm}$ for Cs$^+$

Nitrogen

5 microns
NanoSIMS is complementary to Transmission Electron Microscopy

NanoSIMS has:

- Lower spatial resolution (50 nm vs. 1 nm)
- Higher sensitivity
- Capability to measure isotopes

NanoSIMS ion image of a sectioned *B. thuringiensis* spore
NanoSIMS enables precise quantification

NanoSIMS: Flat-top peaks

Energy Dispersive X-ray spectrum
Isotopic Measurements in Ultra-trace Samples while excluding contaminants

- Trace *Bg* spore samples from Kreuzer-Martin, University of Utah
  - Known isotopic composition

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### Notation

\[
\delta^{13}C \text{ (permil)} = \frac{(^{13}C/^{12}C) - (^{13}C/^{12}C)_{\text{stnd}}}{(^{13}C/^{12}C)_{\text{stnd}}} \times 1000
\]

**Example of imaged area**

Sample UU182 (Reference)  
Sample UU234 ("unknown")  

- Sample UU234 "True" 
  - (n=29)
Trace element composition at single particle level

- Same answer as ICP-MS with $10^6$ fewer spores
- Can extract signature of agent only

**Sr/Ca**
Sample concentration
10 to 100 ppm Sr

**Mn/Ca**
Sample concentration
20 to 1000 ppm Mn

**Trace Samples**
Conventional analyses: $10^6$ to $10^9$ spores
NanoSIMS: 1 to 100 spores

(#) = number of particles analyzed
We can resolve bulk signature from micro-signatures

- High resolution NanoSIMS data reveals Fe/Si relationship in mineral coating for two G-medium grown Bti samples

Bulk and high resolution data for the LLNL/DHS challenge samples
Sample Prep: We are careful to avoid methods that alters elemental composition

- Water is useful in dispersing samples
- Ultramicrotomy requires infiltration with $\text{H}_2\text{O}$, acetone, ethanol, other

> Water can remove anions and cations from spores

**Single *Bti* spore data comparing dry and water dispersion**

<table>
<thead>
<tr>
<th>Dispersion method</th>
<th>F/C</th>
<th>Si/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>dry</td>
<td>0.015</td>
<td>0.0015</td>
</tr>
<tr>
<td>water</td>
<td>0.010</td>
<td>0.0005</td>
</tr>
</tbody>
</table>

Means and 95% confidence interval
Focused Ion Beam (FIB) is the most conservative sectioning method

- Can work with dry preparations
- Precise sample selection conserves samples and ensures quality
- Top-cuts are comparable in cost to ultramicrotomy

LLNL FEI Novalab 600 Dual-beam FIB-field emission SEM Instrument

Additional capabilities

- STEM imaging
- Pt, C & Au deposition
Focused Ion Beam (FIB): Top-cut

- A representative sample prepared relatively rapidly (~20 per ½ day)
- SEM imaging provides high degree of control on quality

Sample deposited on high purity gold foil on aluminum substrate

Low angle FIB cut to remove top

Section ready for NanoSIMS analysis
NanoSIMS Results: Quantitative Digital Images

• Data processed pixel by pixel with custom software

Oblique view of FIB section of G-medium, agar-grown spores, with area of analysis.

NanoSIMS ion images for sectioned *Bti* spores
We can quantify microstructural signatures

<table>
<thead>
<tr>
<th>Analysis type</th>
<th>G-Fermentor</th>
<th>G-Agar</th>
<th>NB-Agar</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>062104G-F-SDS-A (D)</td>
<td>090204G-A-H2O-L (A)</td>
<td>101804G-A-Casc-L (F)</td>
</tr>
<tr>
<td></td>
<td>101804G-A-Casc-A (G)</td>
<td>101804NB-A-Casc-L (H)</td>
<td></td>
</tr>
</tbody>
</table>

Total silicon in spores

- D4: Analysis type (6)
- A4: Analysis type (5)
- F4: Analysis type (7)
- G4: Analysis type (11)
- H4: Analysis type (13)

Analysis type:
- top-cut
- whole, dry
- whole, H2O
We can differentiate added Si from native Si

Weaponized surrogate

Weaponized

Enhanced

Native
We are building a database of signatures
We can quantify core and coat signatures to differentiate samples

- High sensitivity of NanoSIMS provides a clear picture of microstructural signature variability

RSD = 50%, 40%

RSD = 20%, 60%

RSD = 90%, 100%

TEM threshold ~0.1 wt. %
Elemental signatures: Samples group by elemental composition

Spore prep Mn/Ca (g/g)

Spore prep Fe/Ca (g/g)

media differences

growth method differences

run differences
Elemental Addition Experiments

There is a direct relationship between elemental concentration in media and spores

METHODS
- Add Sr & Ba to sporulation medium (0, 1, 10 & 100 micromoles per liter)

RESULTS
- Order of magnitude differences in elemental concentrations in spores
- Sr and Ba substituting for Ca

Mean, standard error and number of analyses shown
Three ways that mineral coatings could develop on spores — *model developed by Brian Viani, LLNL*

**Pre-lysis**
- Metabolic accumulation from mother cell
- Interspace
- Exosporium
- Spore coat

**Post-lysis**
- Passive diffusion into interspace
- Direct adsorption/precipitation on exosporium/spore coat
- Indirect adsorption onto precipitates

**Post-drying**
- Interspace
- Water

**COLOR KEY**
- Blue - diffused
- Red - adsorbed
- Green - precipitated
Expected response for concentration, adsorption, precipitation

**Concentration**
- Diffusion $\rightarrow$ evaporative concentration in interspace
- $\rightarrow$ precipitation in interspace

**Solubility limit**

**Precipitation** on spore surface(s)

**Adsorption** to minerals precipitated on spore surface(s)
Preliminary Chemical Modeling Results

### Scenario

<table>
<thead>
<tr>
<th>Scenario</th>
<th>Si in Media (mg/L as SiO₂)</th>
<th>Mn per mass spores (wt. %)</th>
<th>Fe per mass spores (wt. %)</th>
<th>Si per mass spores (wt. %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-medium low Si</td>
<td>8</td>
<td>4</td>
<td>0.5</td>
<td>0.039</td>
</tr>
<tr>
<td>G-medium high Si</td>
<td>120</td>
<td>4</td>
<td>0.5</td>
<td>0.6</td>
</tr>
</tbody>
</table>

### DHS/LLNL Media

<table>
<thead>
<tr>
<th>DHS/LLNL Media</th>
<th>Media Si/C by SIMS (g/g)</th>
<th>Est. Si in Media (mg/L as SiO₂)</th>
<th>Mn in spores (wt. %)</th>
<th>Fe in spores (wt. %)</th>
<th>Si in spores (wt. %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-medium</td>
<td>0.02</td>
<td>70</td>
<td>0.1 - 3</td>
<td>0.1 – 1.5</td>
<td>0.02 – 0.14</td>
</tr>
<tr>
<td>NB-medium</td>
<td>0.0025</td>
<td>20</td>
<td>~0.003</td>
<td>0.01</td>
<td>0.1 – 0.16</td>
</tr>
</tbody>
</table>

*Predicted Ca level far exceeds observed Ca levels (not shown)*

*Highest predicted Si:Fe molar ratio is 1.2; NB sample at ~10*
We are studying elemental uptake and loss

- A fundamental factor effecting spore elemental composition
  - From media
  - Post processing
  - Washing
  - Both uptake and loss

- Also potentially useful for dating spores

Initial signature from media

Possible density separation reagents

Growth & stationary phase

Separation

Washing

Drying & additives

Analytes can diffuse in and out of the spore
Elemental uptake is moderately rapid

- Expose *Bti* spores to LiF and CsCl in solution
- Li and Cs incorporated through to spore core in <5 minutes
- At 50% saturation level in ~1 day (vs. ~1 hour for D₂O)

Implication: Post production processing
Conclusions

- **Demonstrated NanoSIMS capability:**
  - Trace element and isotopic analyses
  - Single bioagent particle forensic capability
  - Micro-structural characterization

**Signatures**

- Native mineral coatings observed under various growth conditions
- Elemental composition of spores is proportional to media composition for select elements
- We can differentiate samples by using elemental and isotopic signatures
  1. We can do sample matching
  2. We would need to establish the false-positive/false-negative probabilities for court applications
  3. We are developing models signatures to methods

SAMPLES ARE KEY
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Sutapa Ghosal – Diffusion experiments
Brian Viani – Modeling

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Helen Kreuzer-Martin – spores, bulk stable isotope analyses

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