



Measuring Nano-cytotoxicity Over Time: High Throughput Detection of Cell Death and Cellular Signaling Pathways”

SRC/SEMATECH TeleSeminar Series

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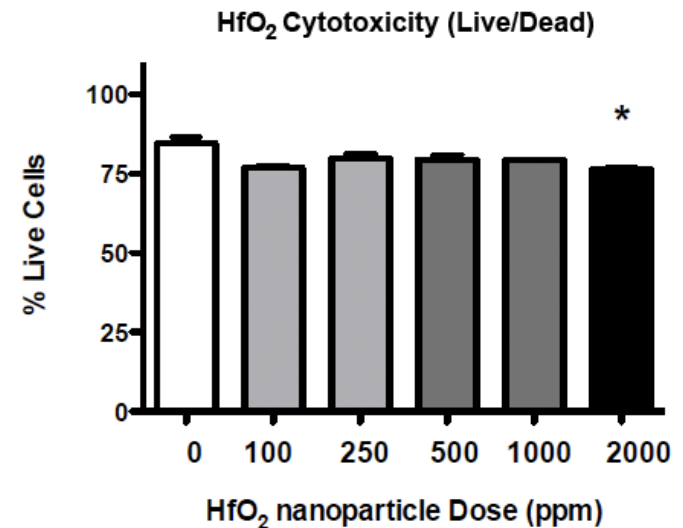
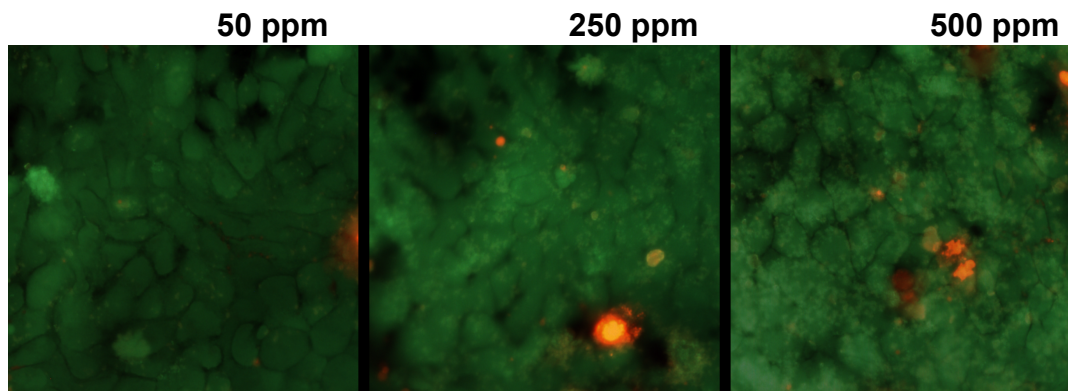
University of Arizona

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Examination of Nano-cytotoxicity of HfO₂: Live/Dead vs Real Time Cell Analysis

Fluorescent Assay:

- Grow Adherent Cells to Confluence
- Introduce Nanoparticle-supplemented Medium for 2 hr
- Evaluate with Fluorescent Dyes
 - Cell Permeant Green Dye (Calcein-AM) = Live Cells
 - Cell Impermeant Red Dye (Ethidium Homodimer) = Dead Cells

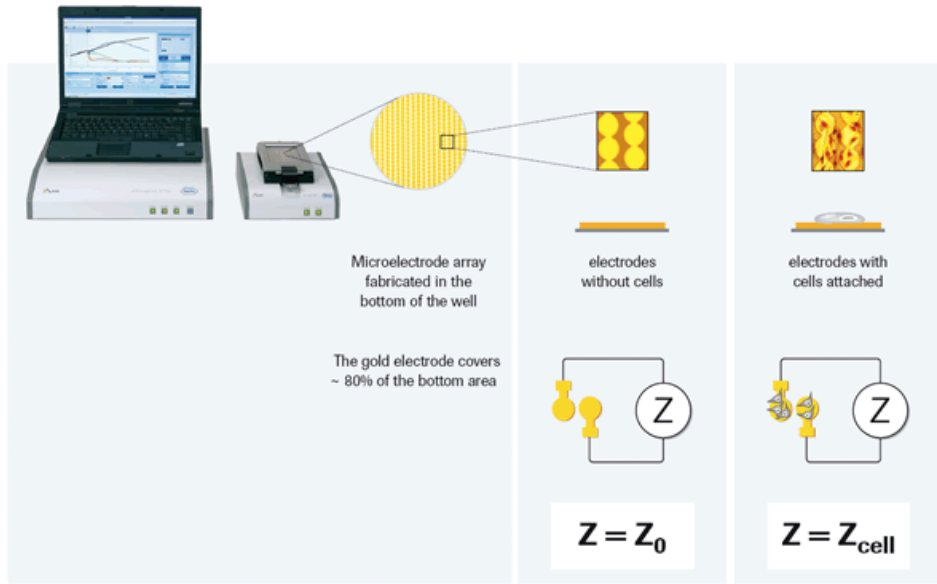


Limitations:

- Single Time Point Response
- Tedious (not easily amenable to High Throughput Screening)

Examination of Nanocytotoxicity of HfO_2 : Live/Dead vs Real Time Cell Analysis

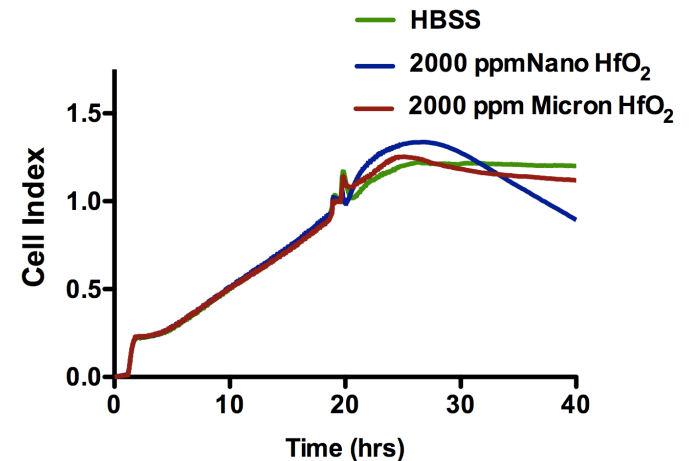
Roche xCELLigence Real Time Cell Analysis (RTCA)



- Non-fluorescence-based assay that measured cell attachment to a membrane
- Cytotoxicity can be monitored as a loss of attachment/reduced impedance

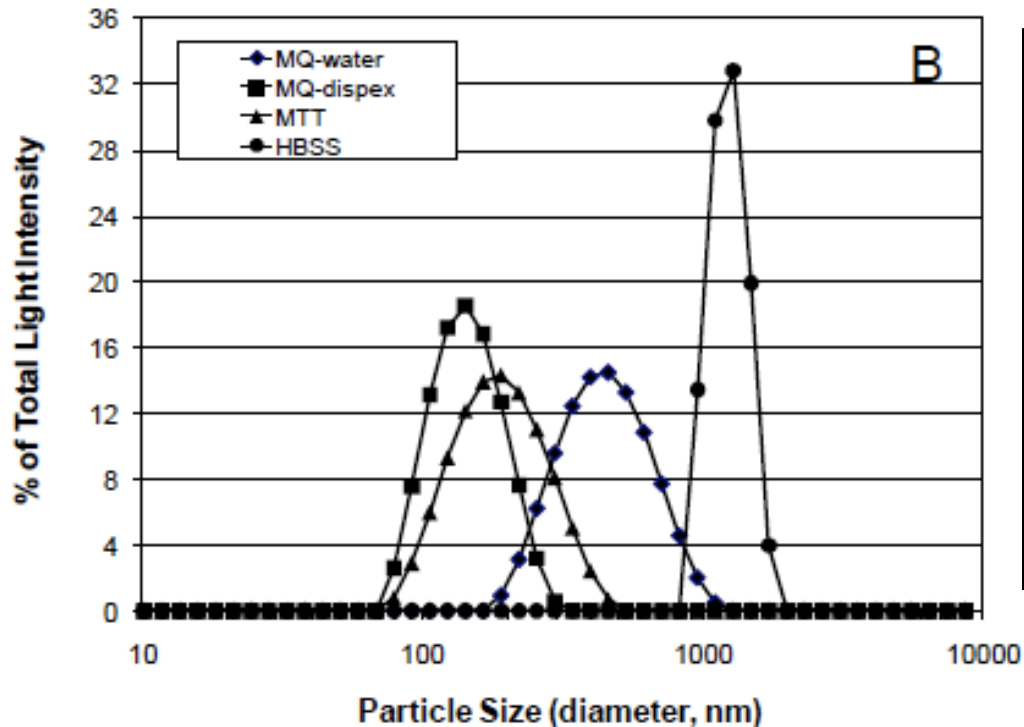
RTCA Experiment:

- Grow Cells to Confluence
- Wash with Nanoparticle-supplemented Medium
- Follow Exposure over time for 8 hrs or more



HfO₂ Particle Distribution in Assay Medium

HfO₂ Particle size distribution (Batch 3) in different media at pH 7.2.

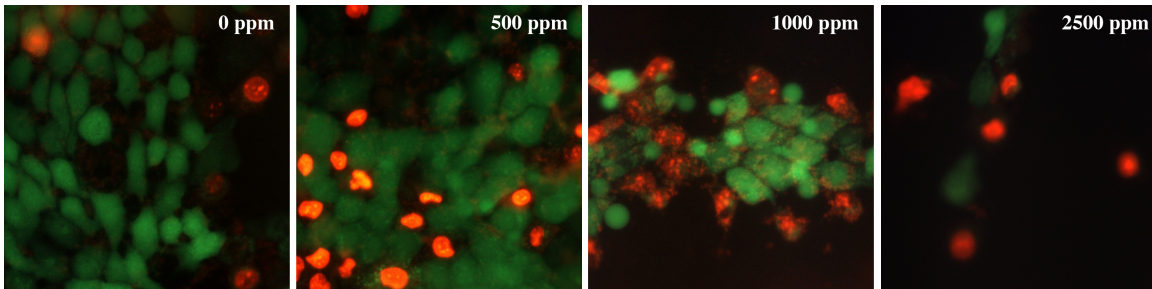


Medium	Average Particle Size
MilliQ Water	350 ± 12
MilliQ Water with Dispex	139 ± 2
MTT Assay Medium	284 ± 2
HBSS	3242 ± 270

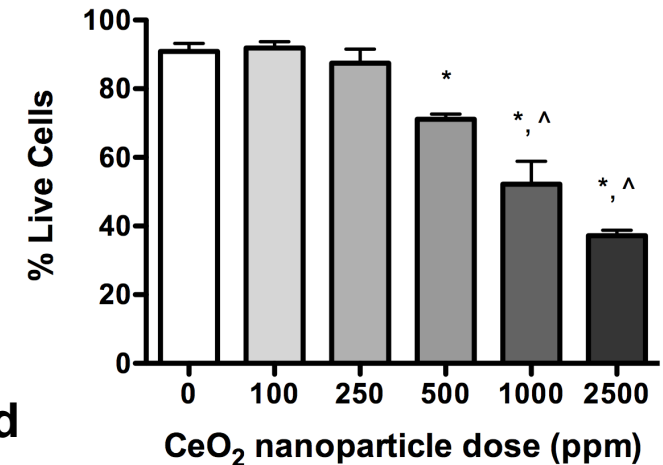
- Assay medium can greatly affect the particle size
- HBSS medium displays the greatest agglomeration
- Repeat RTCA experiments in PBS

CeO₂ Nanoparticle Cytotoxicity

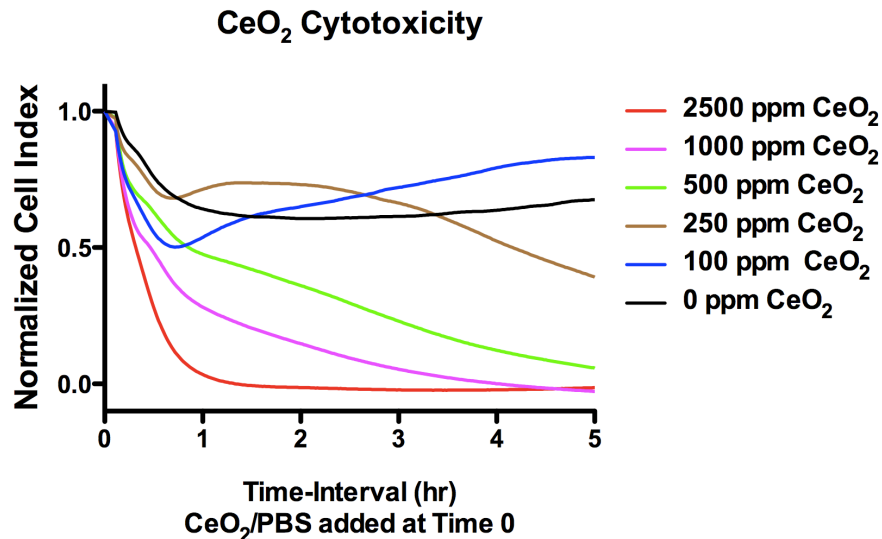
Live/Dead Assay—2 hr in PBS



CeO₂ (<25nm) Cytotoxicity



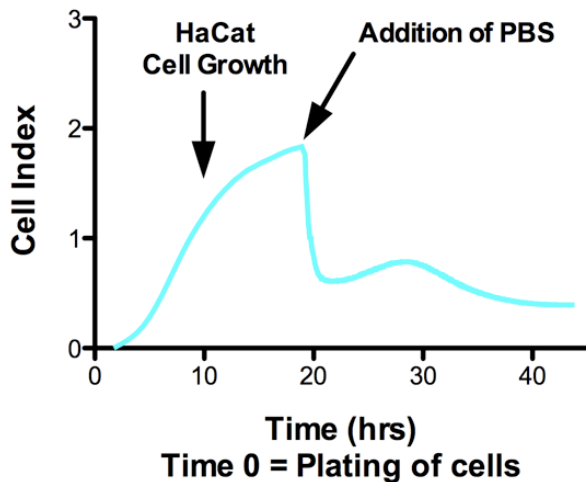
RTCA Impedance Assay--5 hr in PBS and Normalized



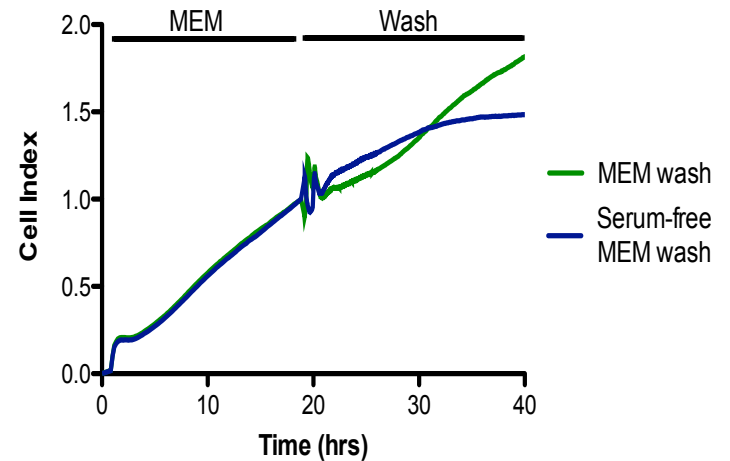
- CeO₂ displays more toxicity than HfO₂ in both assays
- Good Fit for data from two assays--clear shift in cytotoxicity at 500 ppm CeO₂
- Cells appear to recover impedance after 45 min and low dose CeO₂
- Extended incubations suggest 500 ppm CeO₂ is extremely cytotoxic
- Note loss of cells in Live/Dead (RTCA?)

PBS Contribution to RTCA Assay

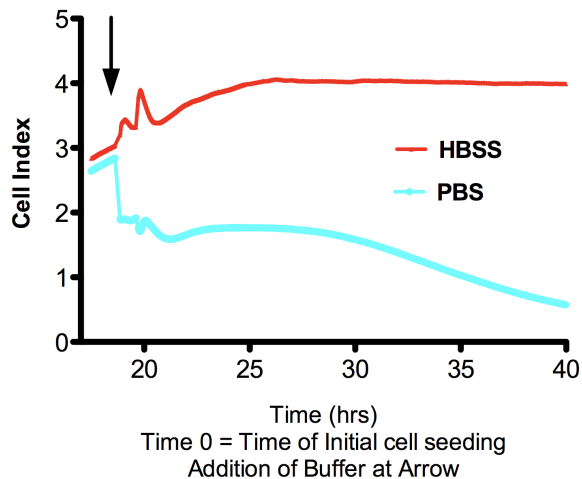
PBS Induced Impedance Changes



Solution Exchange to PBS Results in a Large Impedance Change



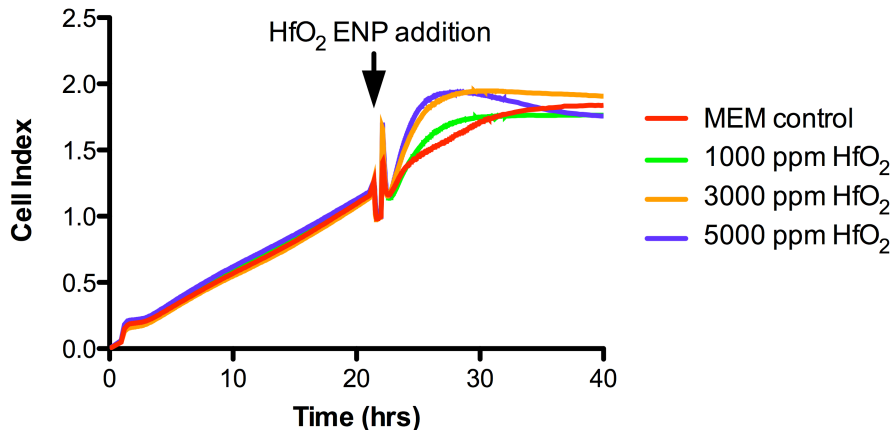
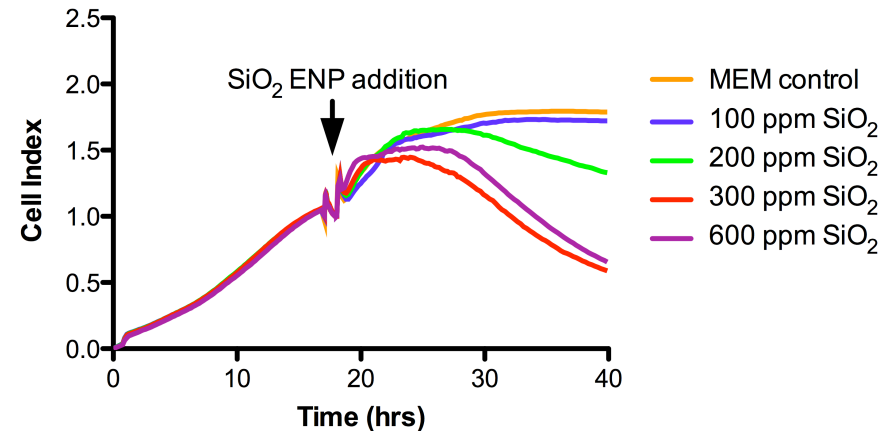
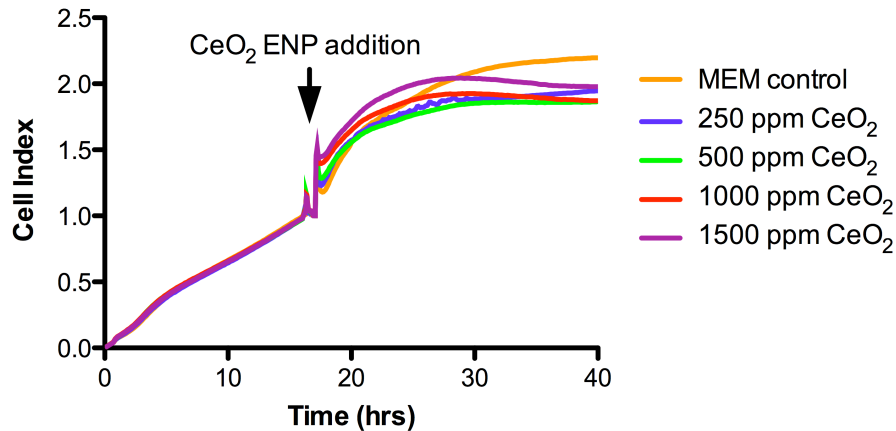
RTCA Impedance upon Buffer Change



Solution Exchange to HBSS Does not Significantly Alter Impedance Measurements (16HBE14o- cells)

Solution Exchange to MEM Does not Significantly Alter Impedance Measurements (16HBE14o- cells)

RTCA Experiments with 16HBE14o- cells and in Minimal Essential Medium

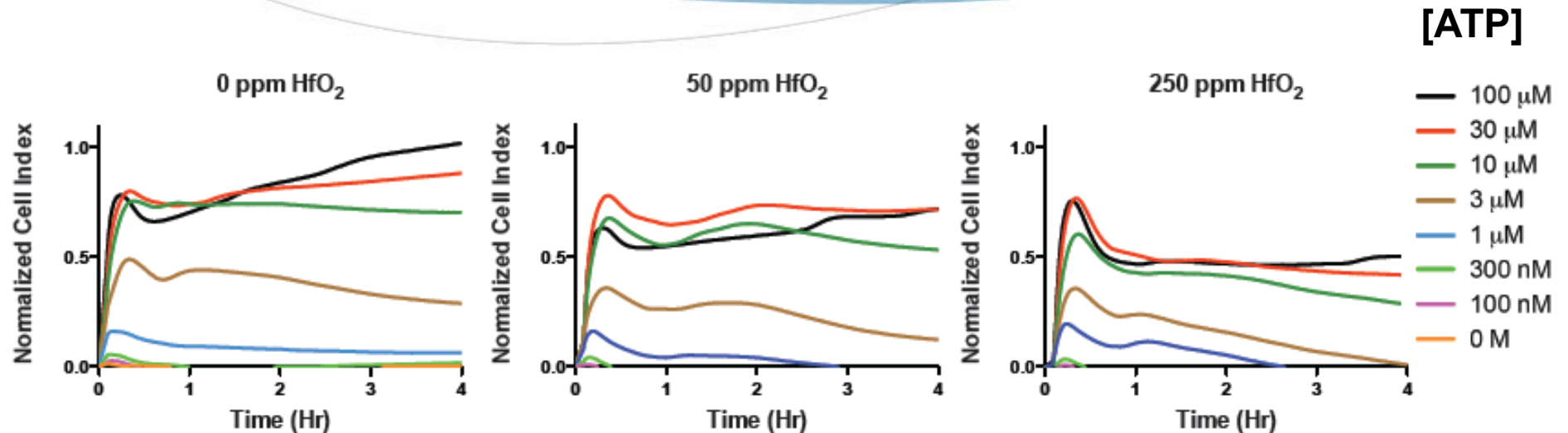


- CeO₂ and HfO₂ nanoparticles displayed no apparent toxicity to 16HBE14o- cells
- SiO₂ nanoparticles induced cytotoxicity at concentrations as low as 200 ppm (developing 10 hrs after application), with greater cytotoxicity at 300 and 600 ppm
- HfO₂ appears to induce an increase in impedance upon application -- indicative of a cellular response

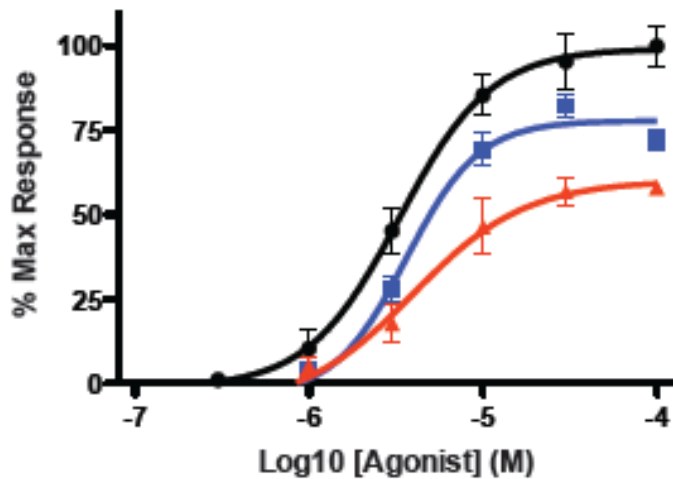
Measuring Activation of Cellular Signaling Pathways with RTCA

- **Measurement of Cell Death is the “Ultimate” End Point for Toxicity Experiments**
- **Many Toxic Effects can Occur in the Cell in the Absence of Cell Death**
 - **Cell Transformation – e.g., Cancer**
 - **Loss of Ability to Respond to Cellular Signals or Stress**
- **Activation of Plasma Membrane Receptors in Adherent Cells can Initiate Cellular Signaling Pathways (e.g., Ca²⁺ signaling) that Alters Cell Spreading and Adhesion**
- **These changes can be measured with RTCA as distinct impedance changes**
- **We used the RTCA to evaluate low level Nanoparticle exposure on ATP-induced airway epithelial cell signaling**
 - **Loss/Reduction of ATP Signaling is Associated with Innate Immune Impairment and Chronic Lung Diseases**
 - **We Evaluated whether 24-hr Exposure to Low Doses (i.e., sub-cytotoxic) of HfO₂ Nanoparticles could Alter ATP-induced signaling**

Nanoparticle Effects on Autocrine/Paracrine ATP-signaling in Airway Epithelium



Dose Response Curve

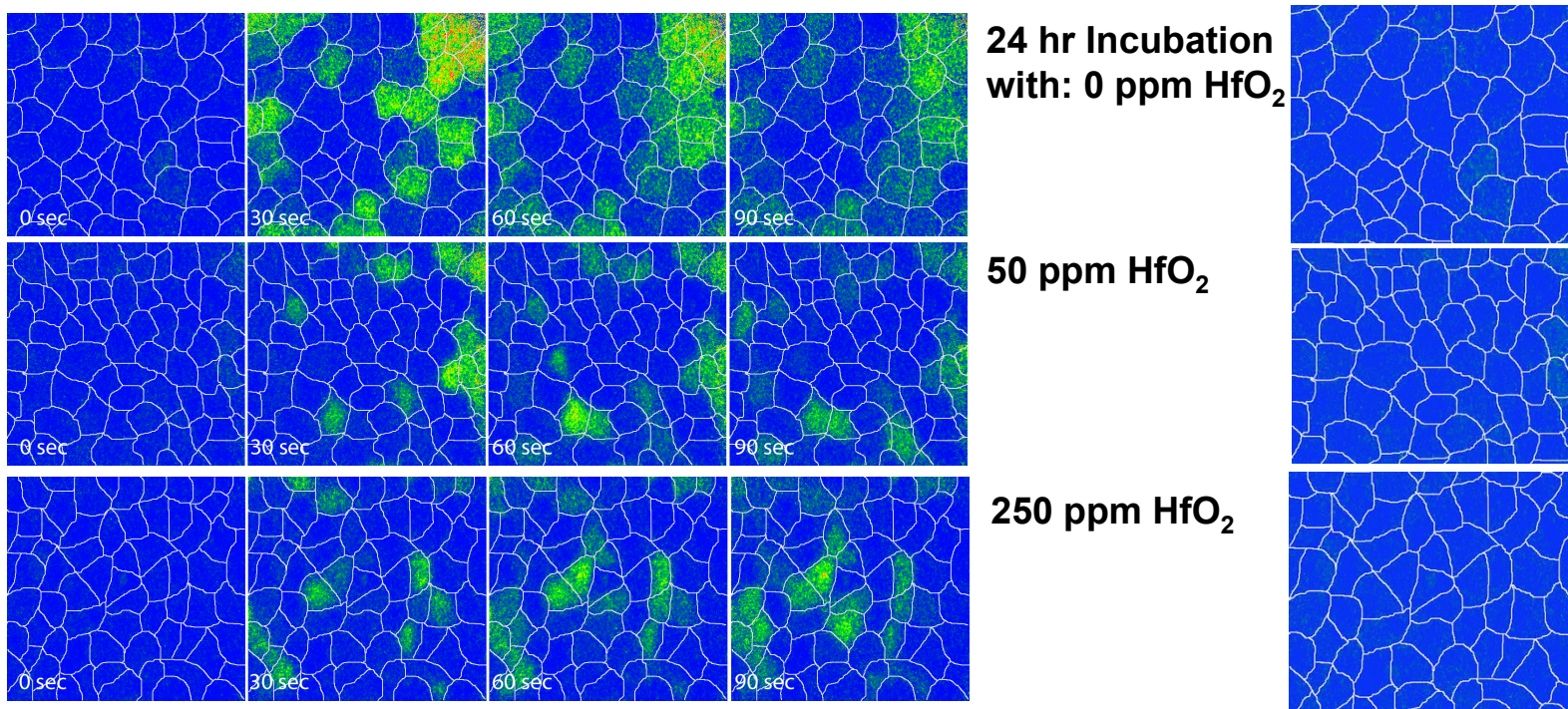


Conclusions:

- 24-hr incubation with HfO₂ inhibits ATP Response at “sub-cytotoxic” levels
- Loss of ATP Signaling is Indicative of Toxicity
- Alterations of Signaling Pathways can Contribute to Chronic Disease

Digital Imaging Microscopy of ATP-Induced Second Messenger Signaling (Ca^{2+})

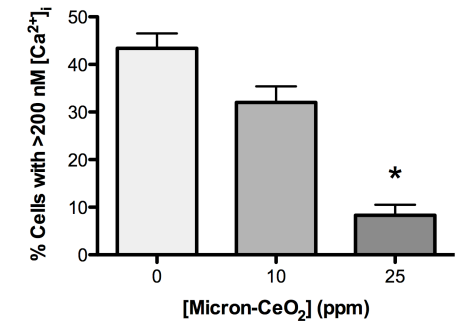
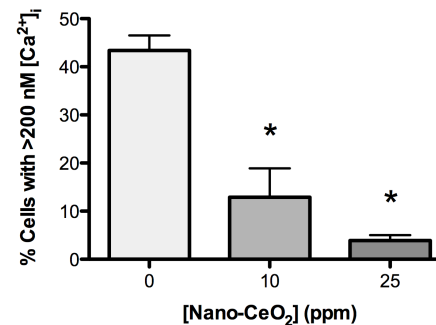
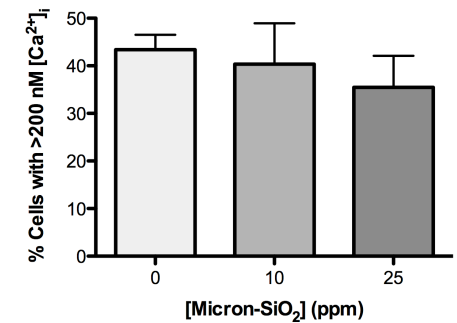
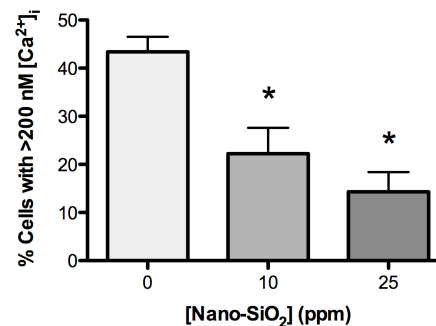
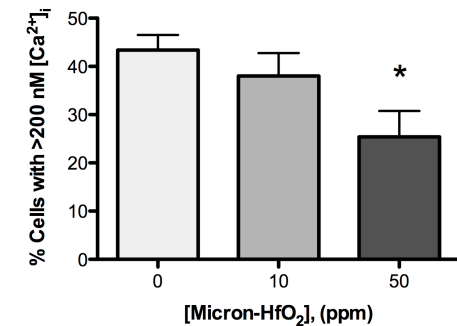
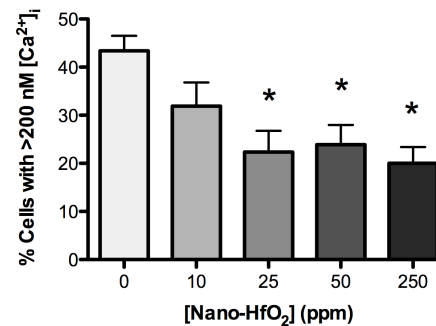
- One Signaling Pathway Downstream from ATP Activation is the increase in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$)
- Cells were loaded with a Ca^{2+} -sensitive dye (fura-2) and Mounted onto a Digital Imaging Microscope
- Cells Were Exposed to $1 \mu\text{M}$ ATP (typical signaling concentration in airway epithelium) and assayed for $[\text{Ca}^{2+}]_i$ changes over 3 minutes



Nanoparticle Effects on ATP-induced Ca^{2+} Signaling

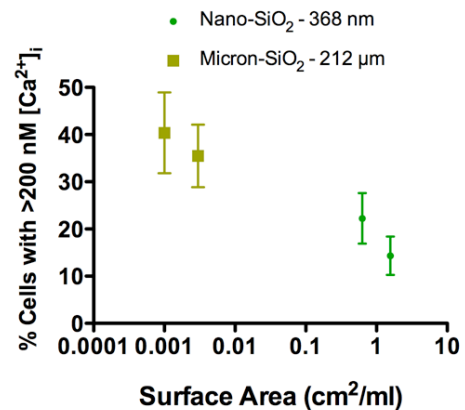
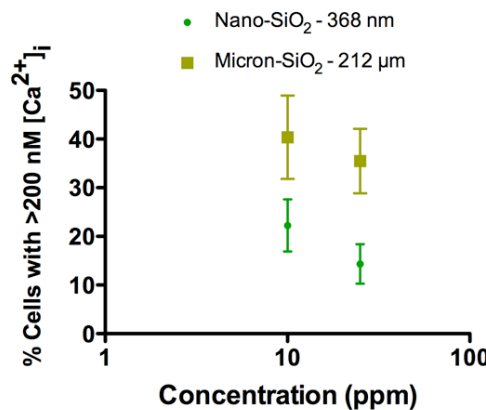
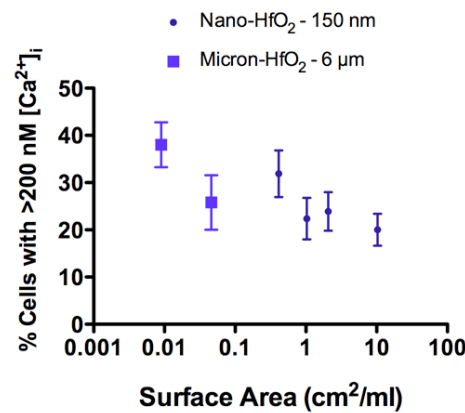
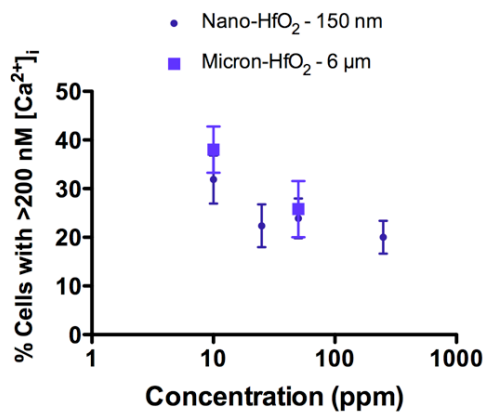
Experiments Evaluating Responses to 1 μM ATP were Conducted with micron- and nano-sized HfO_2 ; SiO_2 and; CeO_2

- Nano-sized HfO_2 reduced the Ca^{2+} signal at lower concentrations than the micron-sized particles
- Only nano-sized SiO_2 reduced the Ca^{2+} signal in the concentration range tested
- Nano-sized CeO_2 reduced the Ca^{2+} signal the most among the nano-sized particles, but also had a significant impact in the micron range



Concentration vs Surface Area Ca²⁺ Response Relationships

- What is the Best Metric to Evaluate Toxicity?
- Recalculated Ca²⁺ toxicity using both Concentration and Surface Area



Conclusions:

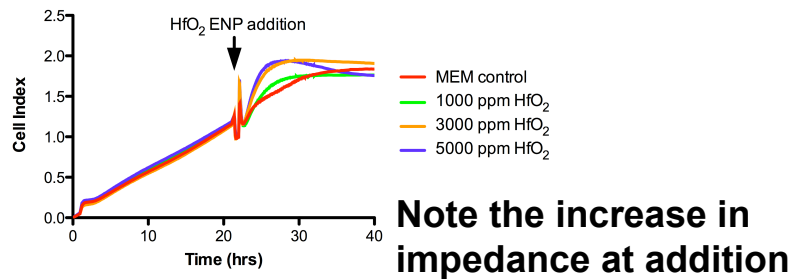
- Both HfO₂ and SiO₂ toxicity (as measured by a reduction in Ca²⁺ Response) display a Logarithmic Relation to Surface Area
- Although Particles labeled as “Nano” when purchased, particle size peaks (noted in Legends) > 100 nm; further work is needed on “Nano” sized particles to observe if Surface Area Relationship holds

Next Steps/Discussion

1) MEM is a good medium for nanotoxicity studies

- Relatively good dispersal
- Relatively good impedance
- Cell growth not compromised over many hours
- Do supplemental amino acids provide a corona?

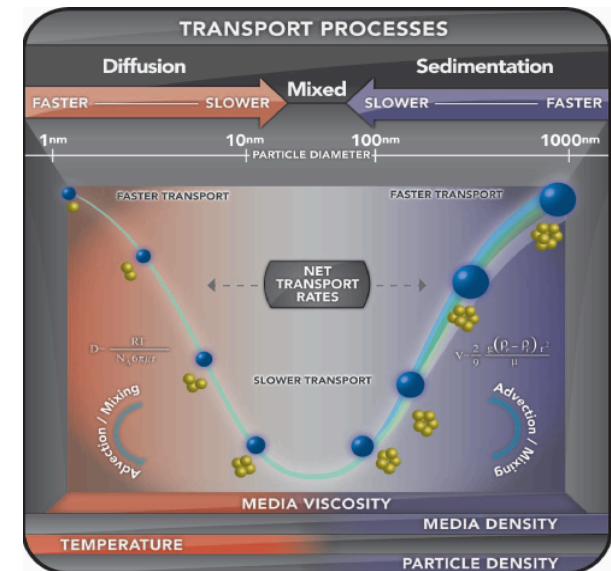
2) Do Nanoparticles Induce Signaling upon Exposure?



3) What else is to be considered to calculate effective Dose?

Figure from: Hinderliter, P.M., et. al. 2010. *Part Fibre Toxicol.* 7:36

4) What Endpoints are Most Appropriate for Toxicology Studies? (not cell death)





Collaborations:

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