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

SRC/SEMATECH TeleSeminar Series

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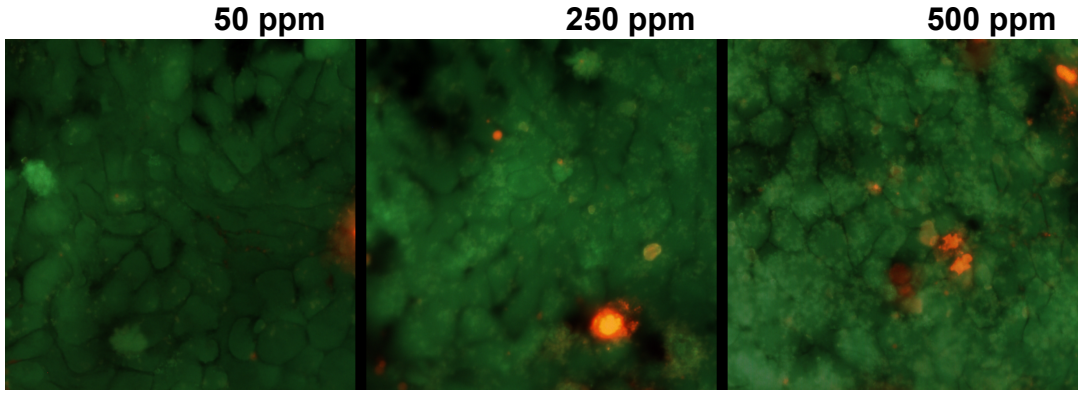
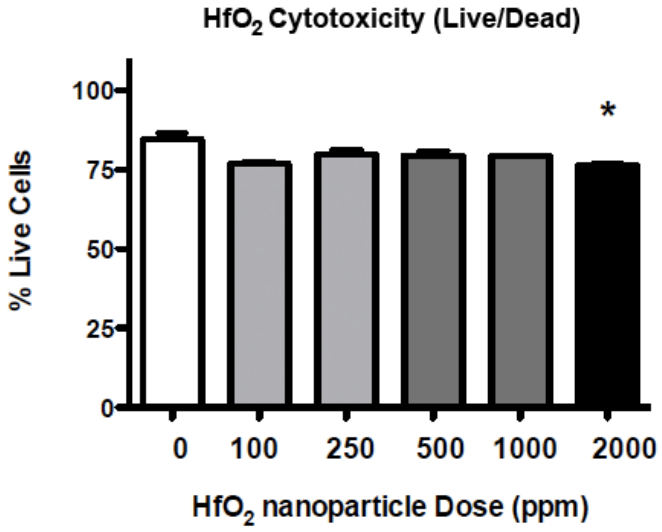
April 21, 2011

Examination of Nano-cytotoxicity of HfO_2: 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



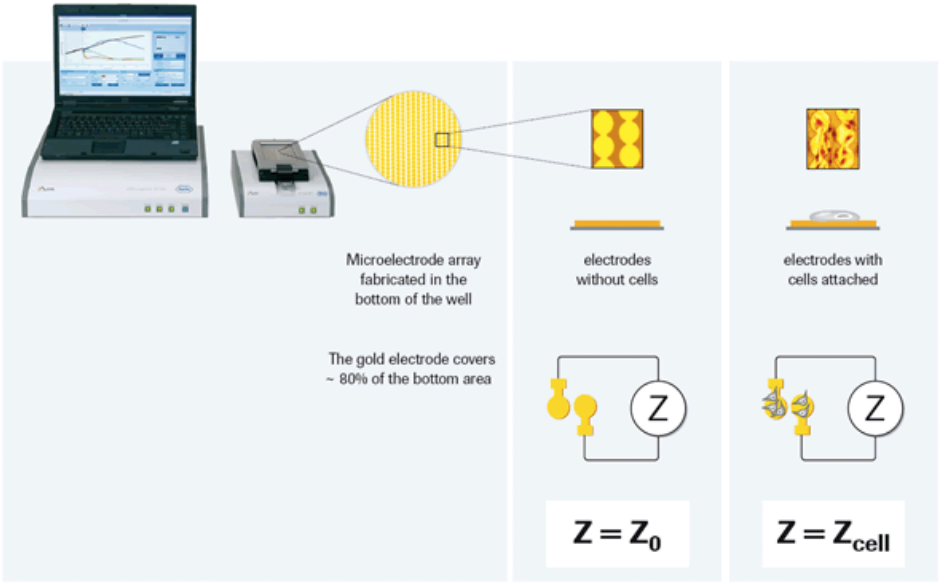


Limitations:

- 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

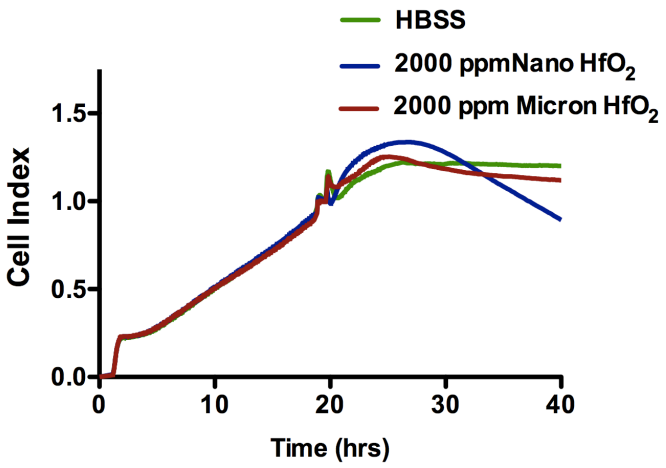
Roche xCELLigence Real Time Cell Analysis (RTCA)



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

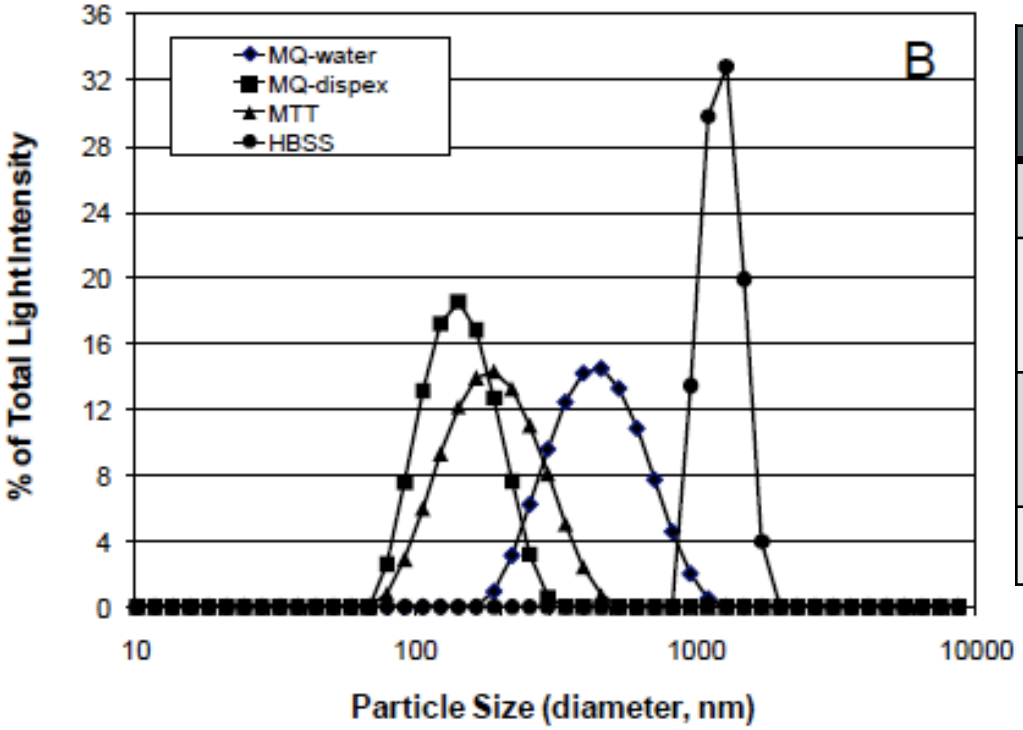
RTCA Experiment:

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



HfO_2 Particle Distribution in Assay Medium

HfO_2 Particle size distribution (Batch 3) in different media at pH 7.2.

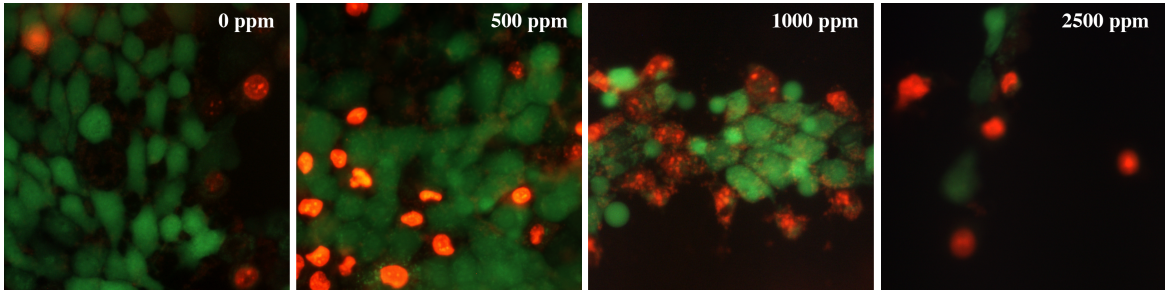


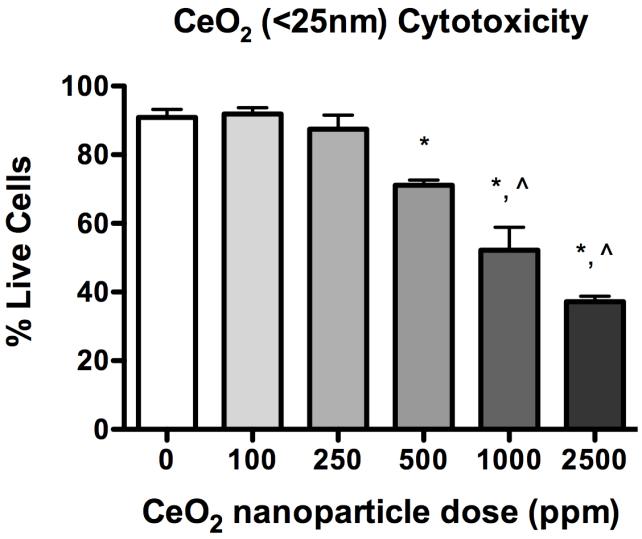
Medium	Average Particle Size
MilliQ Water	350 ± 12
MilliQ Water with Dispex	139 ± 2
MTT Assay Medium	284 ± 2
HBSS	3242 ± 270

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

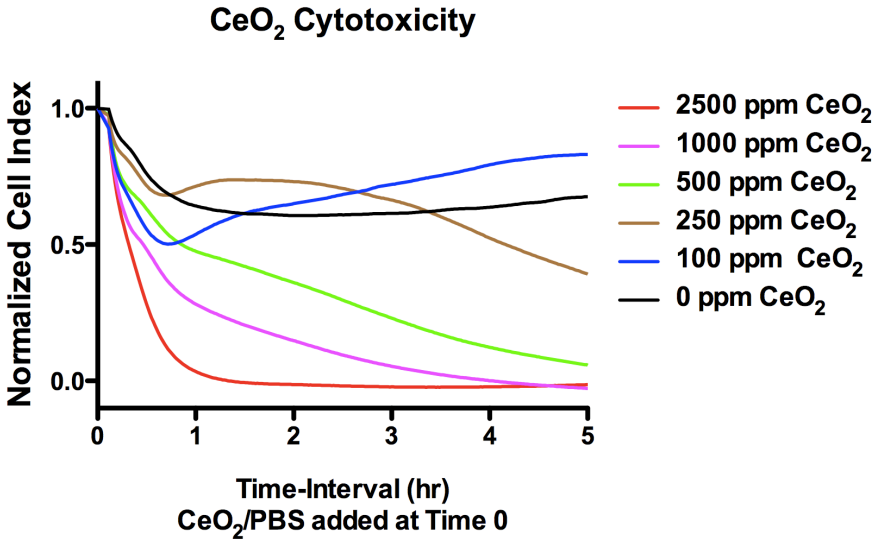
CeO_2 Nanoparticle Cytotoxicity

Live/Dead Assay—2 hr in PBS



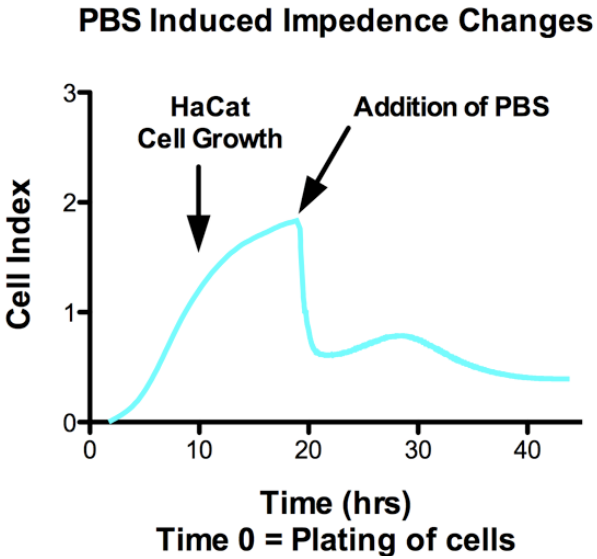


RTCA Impedence Assay--5 hr in PBS and Normalized

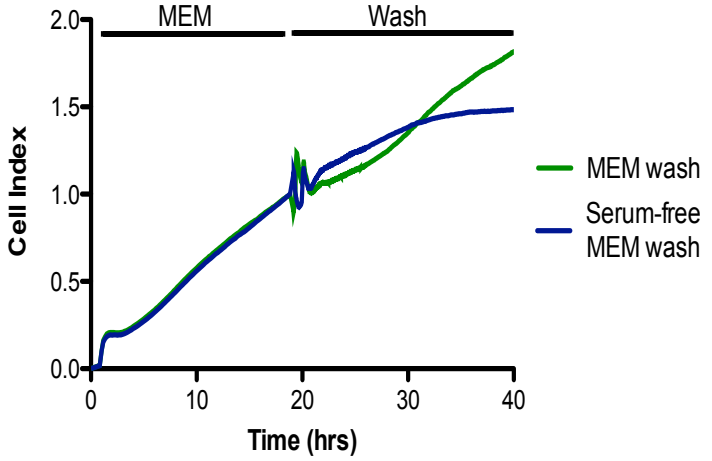


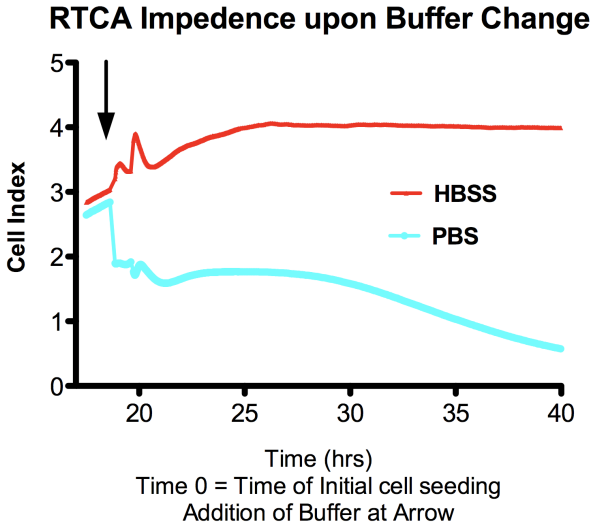
- CeO_2 displays more toxicity than HfO_2 in both assays
- Good Fit for data from two assays--clear shift in cytotoxicity at 500 ppm CeO_2
- Cells appear to recover impedance after 45 min and low dose CeO_2
- Extended incubations suggest 500 ppm CeO_2 is extremely cytotoxic
- Note loss of cells in Live/Dead (RTCA?)

PBS Contribution to RTCA Assay



Solution Exchange to PBS Results in a Large Impedance Change

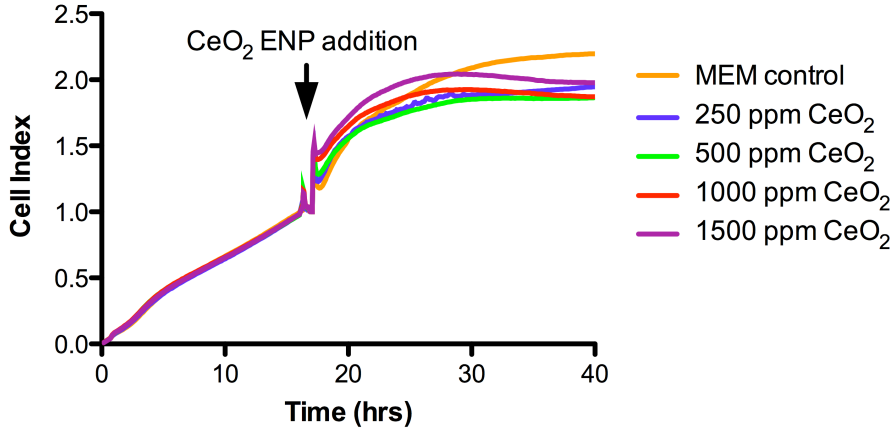


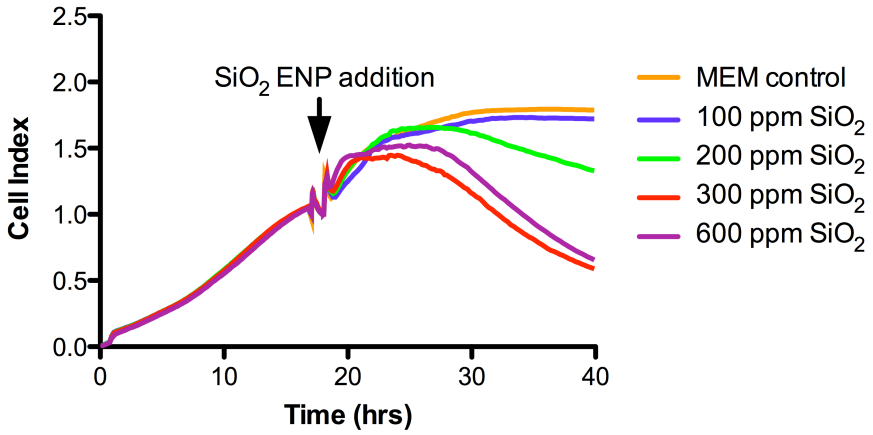


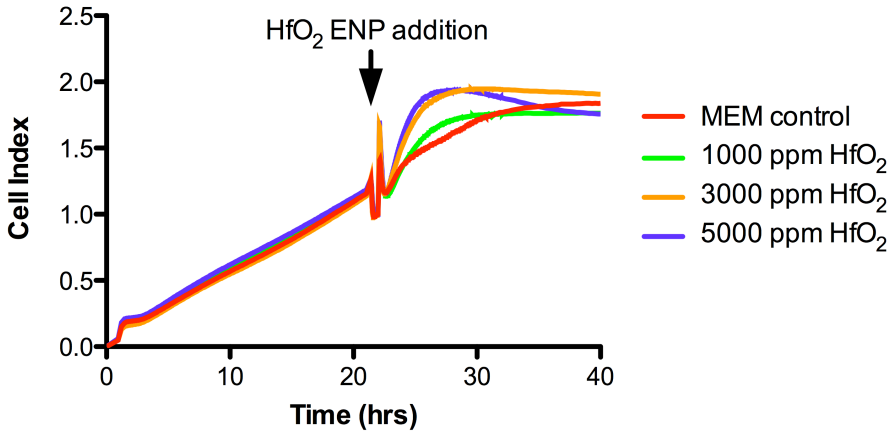
Solution Exchange to HBSS Does not Significantly Alter Impedance Measurements (16HBE14o- cells)

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

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







- **CeO_2 and HfO_2 nanoparticles displayed no apparent toxicity to 16HBE14o- cells**
- **SiO_2 nanoparticles induced cytotoxicity at concentrations as low as 200 ppm (developing 10 hrs after application), with greater cytotoxicity at 300 and 600 ppm**
- **HfO_2 appears to induce an increase in impedance upon application -- indicative of a cellular response**

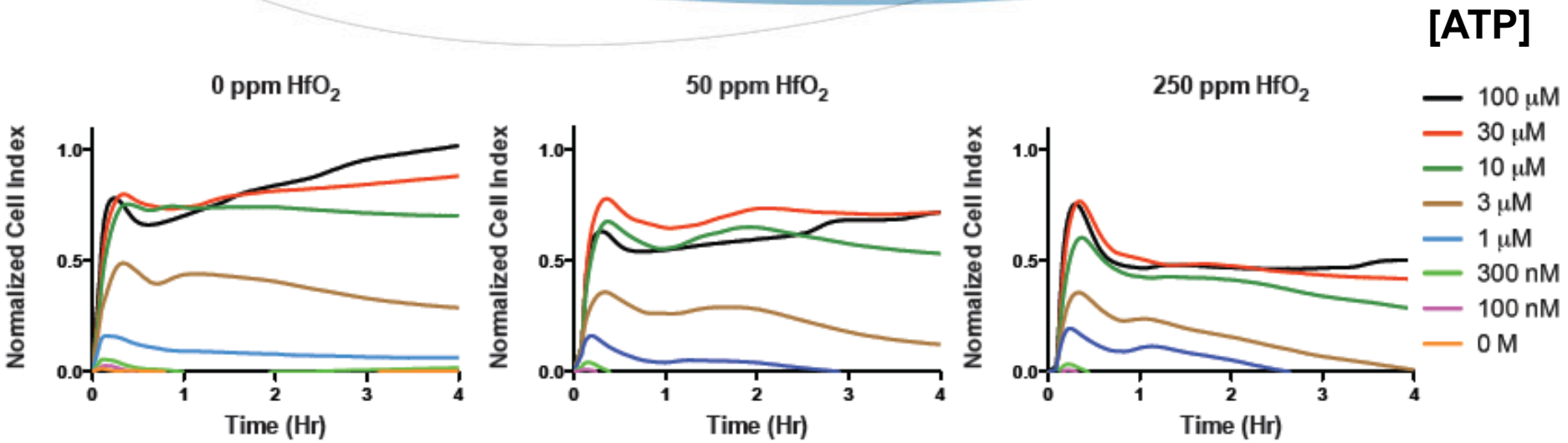
Measuring Activation of Cellular Signaling Pathways with RTCA

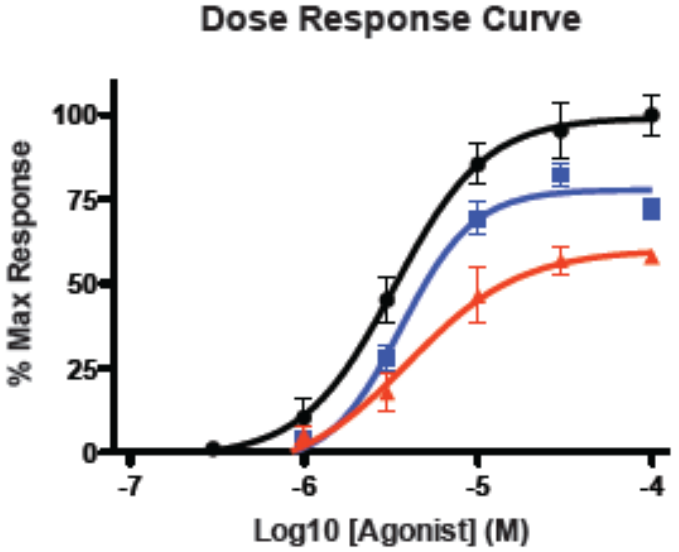
- Measurement of Cell Death is the "Ultimate" End Point for Toxicity Experiments
- Many Toxic Effects can Occur in the Cell in the Absence of Cell Death
  - Cell Transformation – e.g., Cancer
  - Loss of Ability to Respond to Cellular Signals or Stress

- Activation of Plasma Membrane Receptors in Adherent Cells can Initiate Cellular Signaling Pathways (e.g., Ca^{2+} 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 HfO_2 Nanoparticles could Alter ATP-induced signaling

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



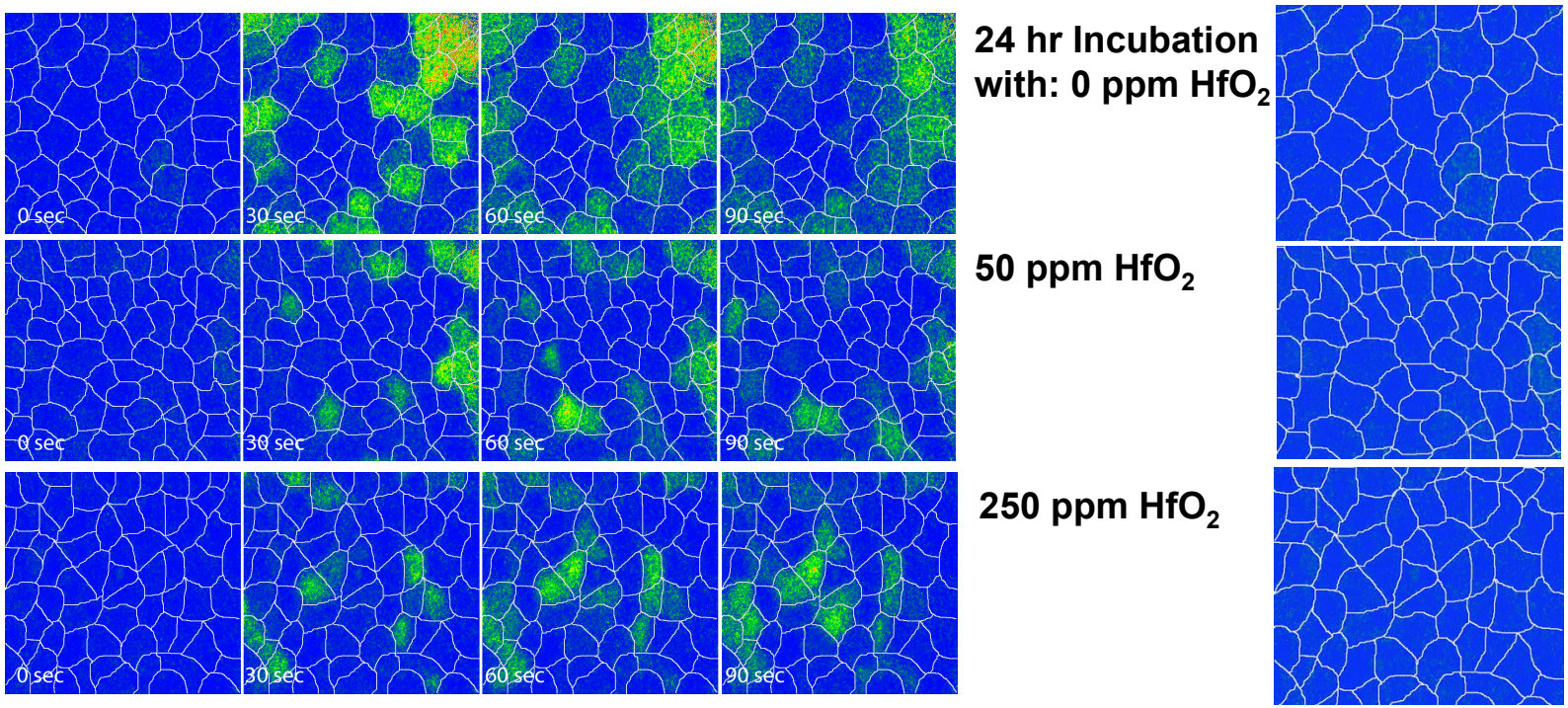


Conclusions:

- 24-hr incubation with HfO_2 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+})

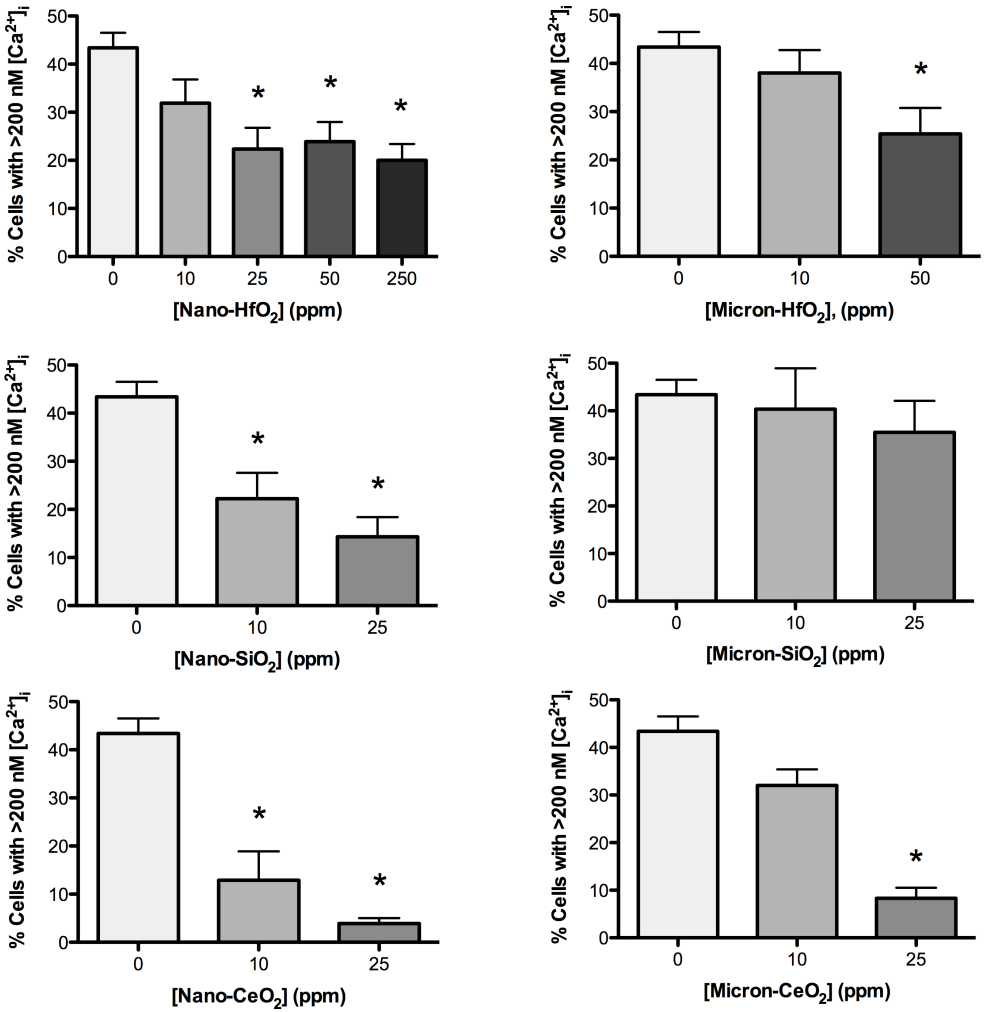
- **One Signaling Pathway Downstream from ATP Activation is the increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)**
- **Cells were loaded with a Ca^{2+}-sensitive dye (fura-2) and Mounted onto a Digital Imaging Microscope**
- **Cells Were Exposed to 1 µM ATP (typical signaling concentration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



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

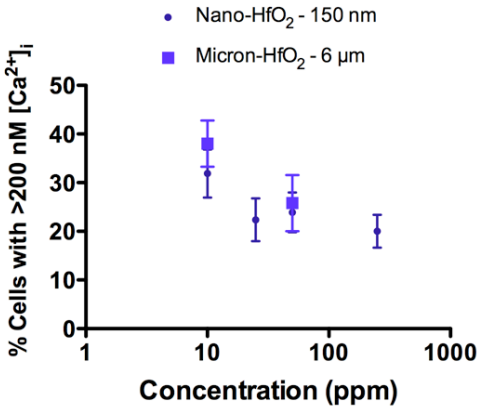
Experiments Evaluating Responses to 1 µM ATP were Conducted with micron- and nano-sized HfO_2; SiO_2 and; CeO_2

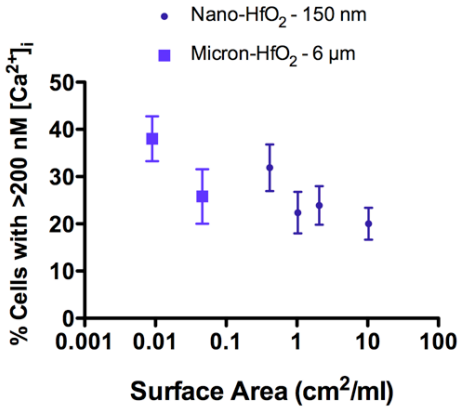
- **Nano-sized HfO_2 reduced the Ca^{2+} signal at lower concentrations than the micron-sized particles**
- **Only nano-sized SiO_2 reduced the Ca^{2+} signal in the concentration range tested**
- **Nano-sized CeO_2 reduced the Ca^{2+} signal the most among the nano-sized particles, but also had a significant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

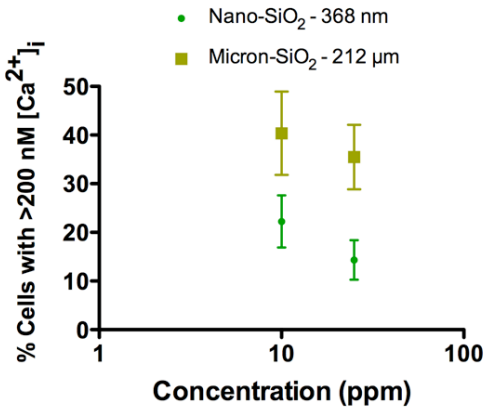


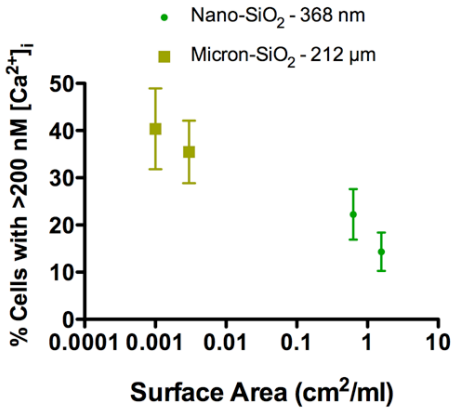
Concentration vs Surface Area Ca^{2+} Response Relationships

- What is the Best Metric to Evaluate Toxicity?
- Recalculated Ca^{2+} toxicity using both Concentration and Surface Area









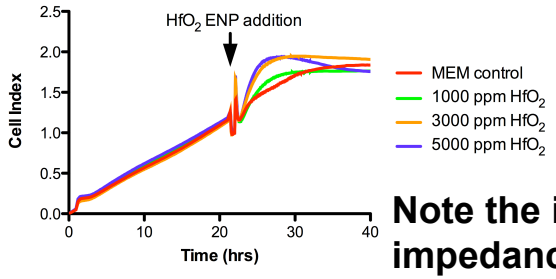
Conclusions:

- Both HfO_2 and SiO_2 toxicity (as measured by a reduction in Ca^{2+} 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?

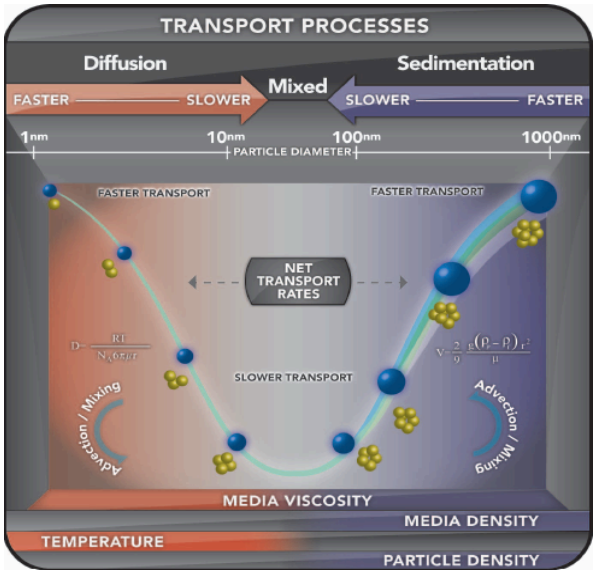
2) Do Nanoparticles Induce Signaling upon Exposure?



Note the increase in impedance at addition

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:

Semiconductor Research Corporation Grant

Jim A. Field (PI)

Reyes Sierra-Alvarez

Lila Otero and Antonia Luna

Farhang Shadman

Buddy Ratner

Graduate Students in the Respiratory Cell Physiology Laboratory:

Mia McCorkel (SRC Fellowship)

Cara L. Sherwood