Toxicity Evaluation of HfO_2 Nanoparticles

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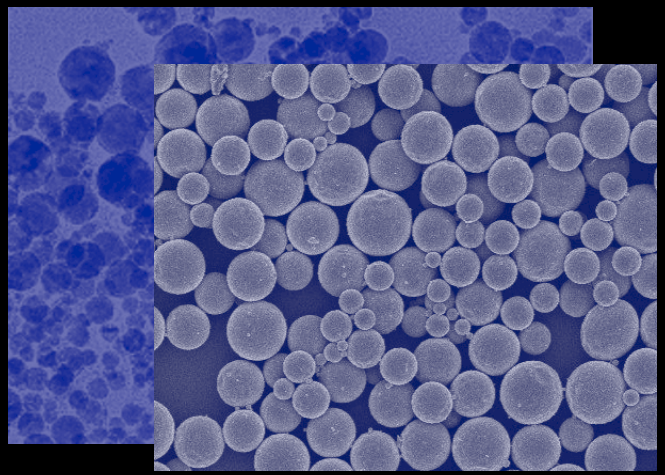
Measuring cytotoxicity of nanoparticles in human cells



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HfO_2 Model Nanoparticles

- **Reference "Micron-sized" HfO_2 particles**
  - Reported particle size: < 44 μm;
  - Measured size (Laser Diffraction and Electron Microscopy; Sierra Group) showed wide range (200 nm-20 μm) with distribution peaks at **500 nm** and **6 μm**
- **Batch 1 nano-sized HfO_2 particles: "Batch-1 360"**
  - Reported particle size: ~20 nm
  - Measured size showed range of 60 nm - 1 μm with a peak at **360 nm**
- **Batch 2 nano-sized HfO_2 particles: "Batch-2 224"**
  - Reported particle size: ~1-2 nm
  - Measured size showed range of 90 nm - 2 μm with a major peak at **224 nm**
- **Batch 3 nano-sized HfO_2 particles: "Batch-3 169"**
  - Reported average particle size: ~100 nm
  - Measured size showed range of 70 - 400 nm with a peak at **169 nm**

Mammalian Cell Models: Epithelium

- We consider two major routes of exposure:
  - Lung (via inhalation or systemic exposure)
  - Skin (via direct contact with nanoparticles)

- Both are lined by epithelial cells that form a barrier to keep outside exposures from underlying cells and tissue

- We use two epithelial cell models for preliminary studies:
  - Human bronchial epithelial cell line (16HBE14o- cells)
  - Human skin epithelial cell line (HaCat)

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Measurements of Cytotoxicity with Fluorescence: Live/Dead Assay

- Cells are incubated with HfO_2 for 2 hr to induce toxicity
- Cells are then exposed to two low-fluorescent compounds: Ethidium homodimer (EtHD-1) and Calcein-AM
- EtHD-1 can only enter damaged cells, where it interacts with DNA and increases its fluorescence 40x (ex/em ~495 nm/~635 nm)
- Calcein-AM can crosses the plasma membrane of live cells. Once inside, the acetyl-methoxy ester groups (AM) are cleaved by non-specfic cell esterases, resulting in charged and highly fluorescent molecules, Calcein (ex/em ~495 nm/~515 nm)
- Net result: In tact, Live, cells fluoresce Green and Dead cells fluoresce Red

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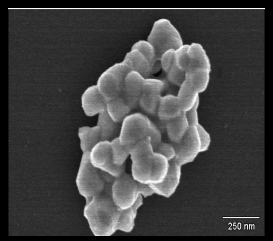
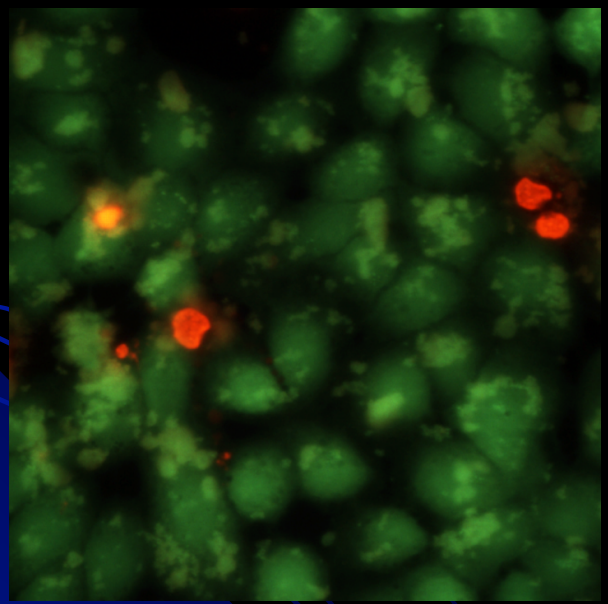
Live/Dead with Micron-sized HfO_2

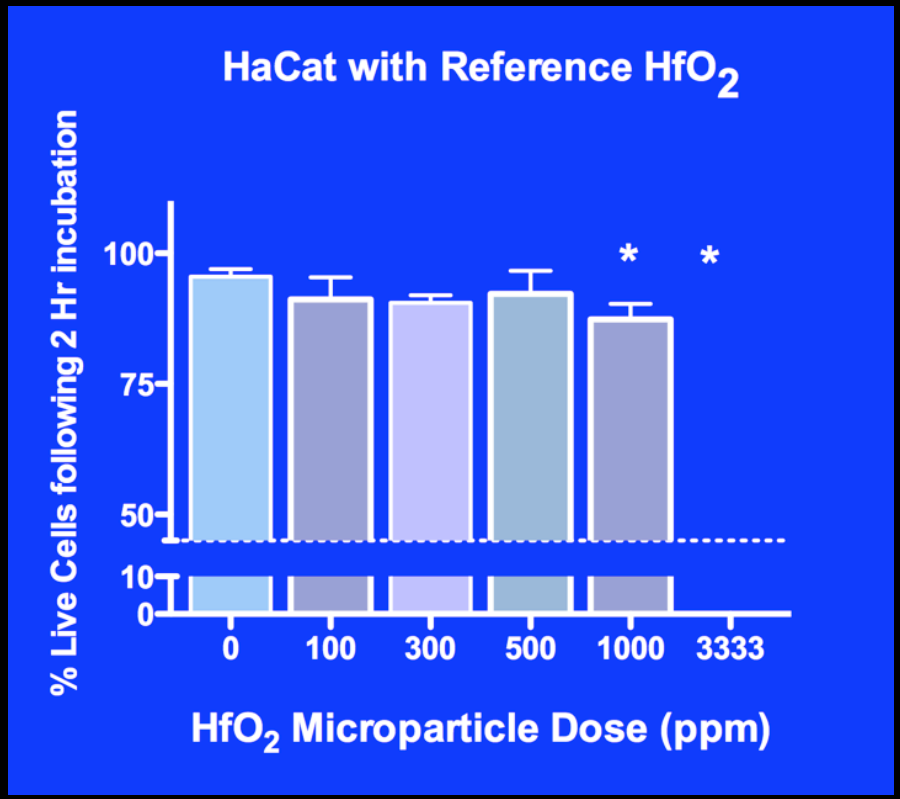


Major size peaks at **500 nm** and **6 µm**

SEM



Live-Dead Fluorescence
(1000 ppm)



- Slight cytotoxicity at 1000 ppm
- Strong cytotoxicity at 3000 ppm
- 16HBE14o- (lung) epithelial cytotoxicity was similar

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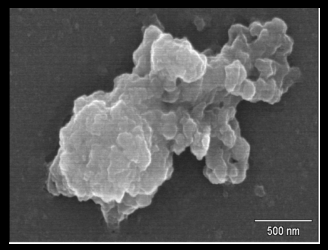
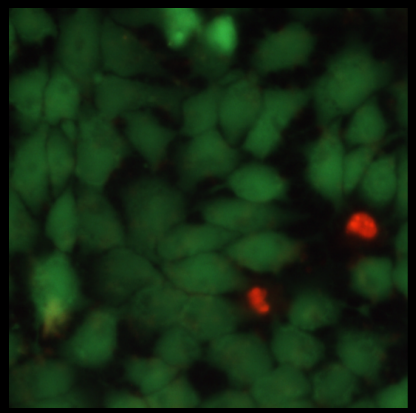
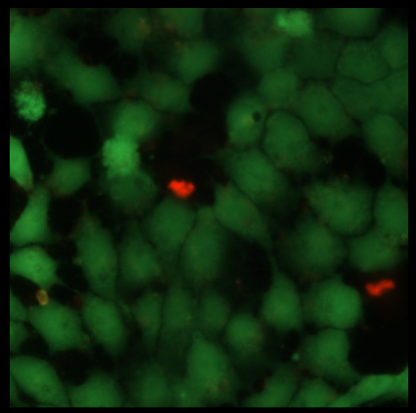
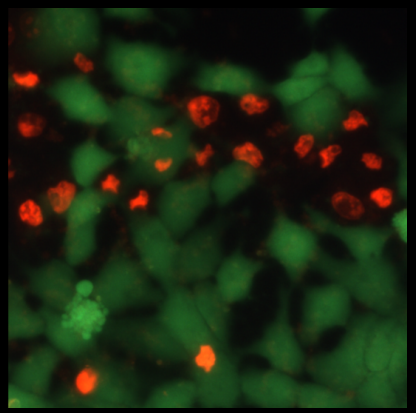
Live/Dead with Batch-1 360 HfO_2

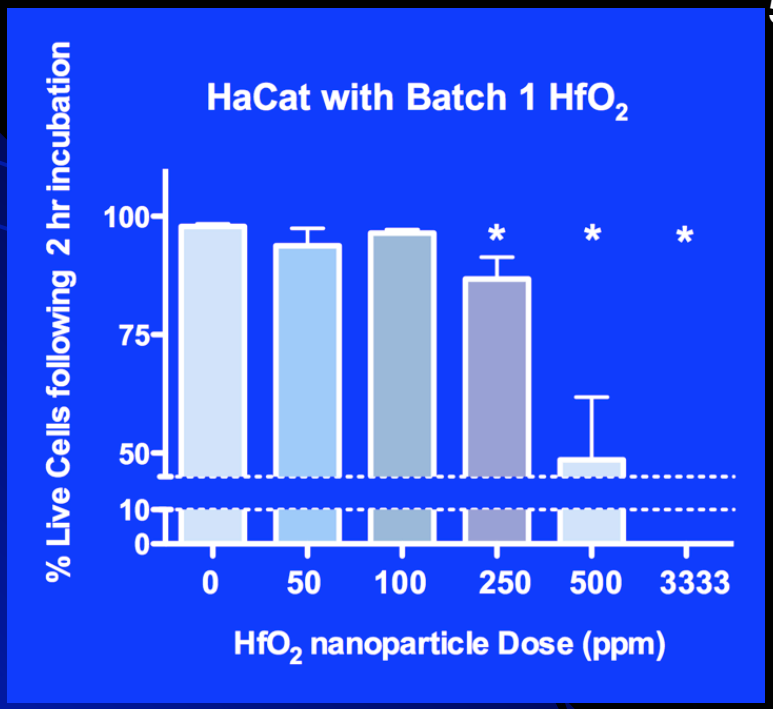






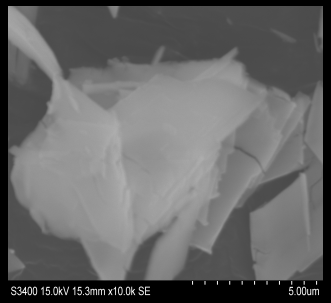


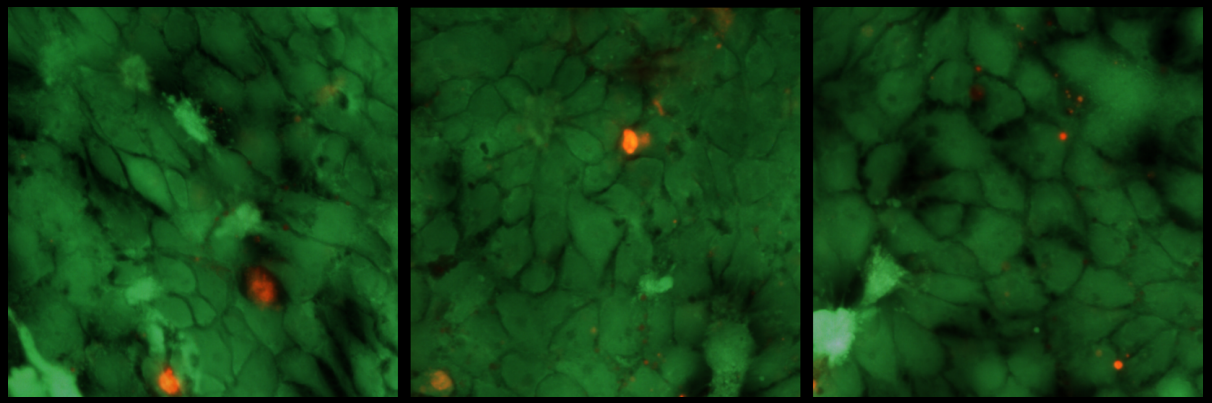
50 ppm 250 ppm 500 ppm



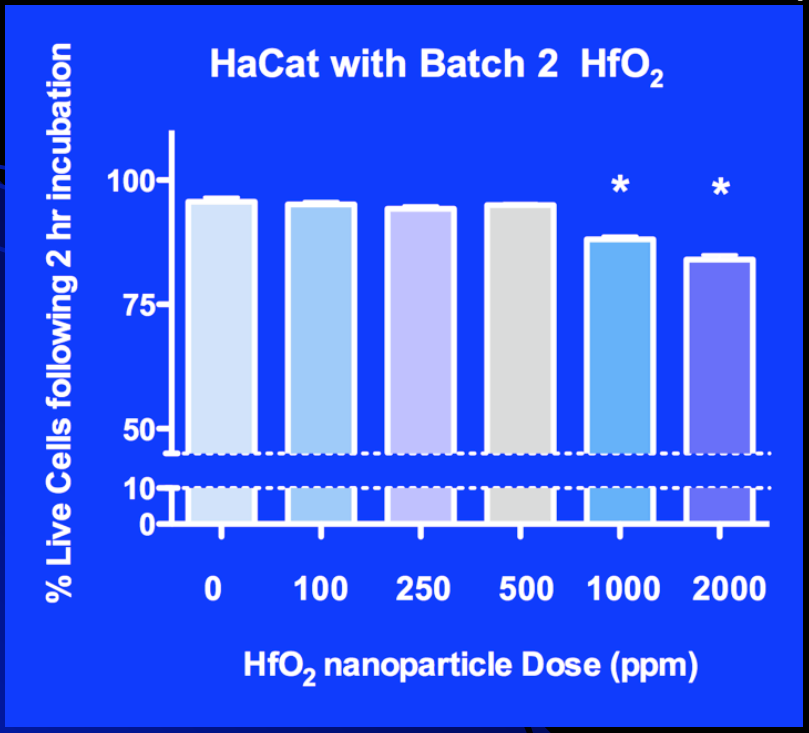
- Slight cytotoxicity at 250 ppm
- Strong cytotoxicity at 500 ppm
- Smaller HfO_2 particles are more toxic to HaCat (skin) epithelial cells
- 16HBE14o- (lung) epithelial cells were similar

Live/Dead with Batch-2 224 HfO_2





50 ppm 250 ppm 500 ppm



- Slight cytotoxicity at 1000 ppm and at 2000 ppm
- Although Batch-2 224 contain smaller particles than Batch-1 336, less cytotoxicity is observed in HaCat (skin) epithelial cells
- 16HBE14o- (lung) epithelial cells were similar

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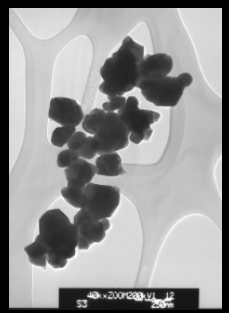
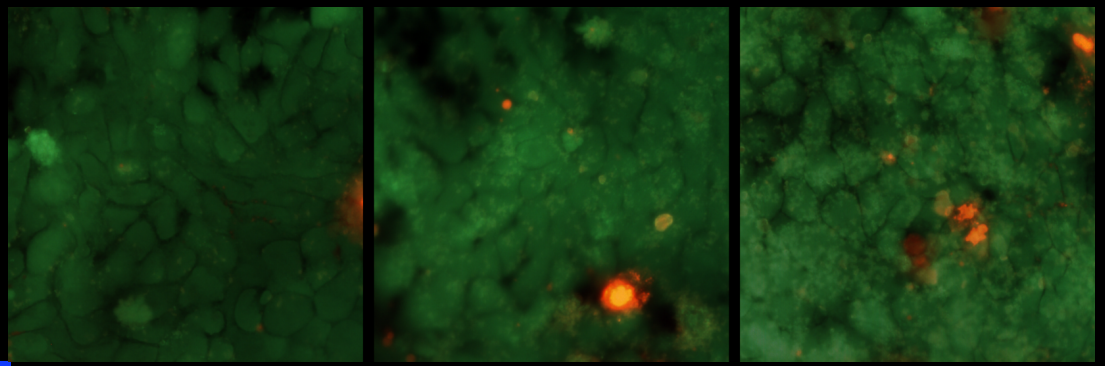
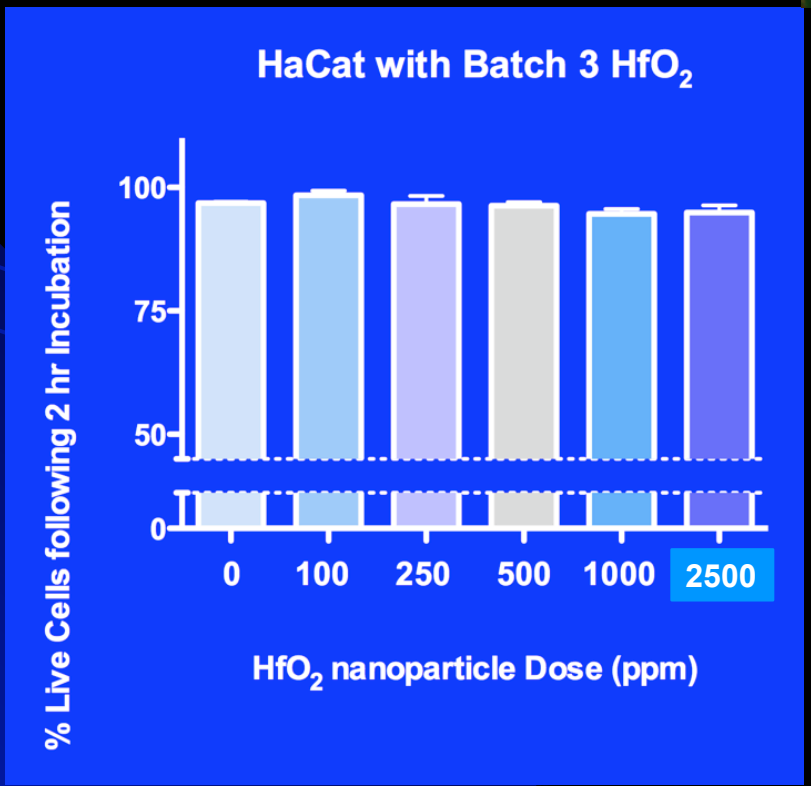
Live/Dead with Batch-3 169 HfO_2





250 ppm 1000 ppm 2500 ppm



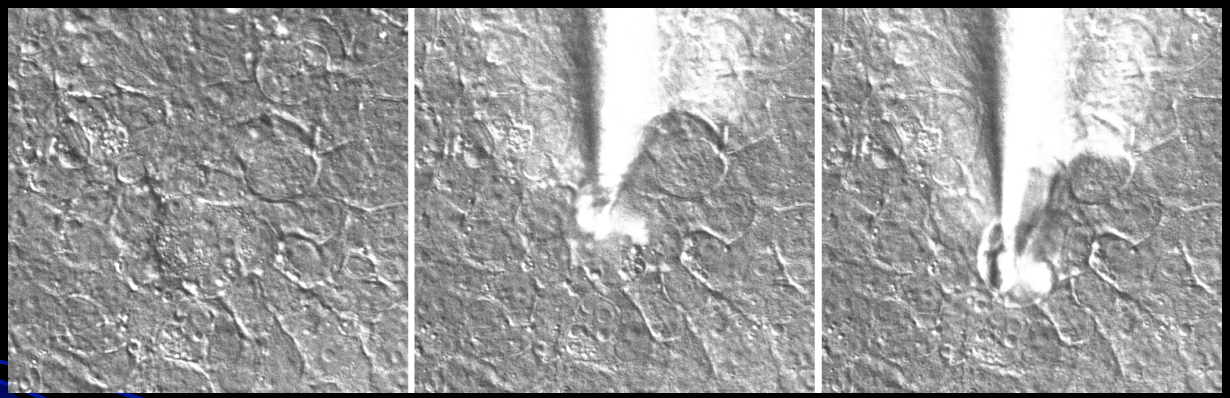
- No cytotoxicity up to 2500 ppm
- HfO_2 particles with the smallest peak size displayed the least cytotoxicity in HaCat (skin) epithelial cells
- 16HBE14o- (lung) epithelial cells not tested

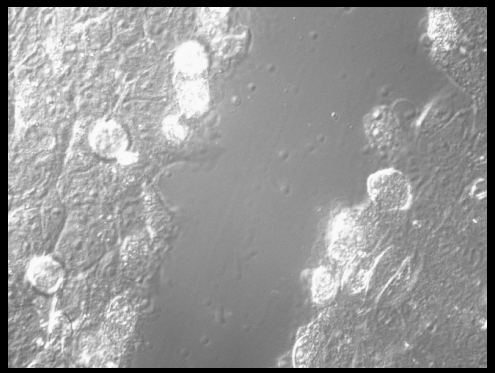
Live-Dead Cytotoxicity Summary

- Live-Dead assay is a straight forward measure for cytotoxicity that works well with mammalian cells
- Similar to Microtox and MTT Assays from Sierra Group, Batch-1 360 HfO_2 displayed highest cytotoxicity
- Smaller HfO_2 sizes did not correlate directly with increased toxicity
- SIMS measurements (Ratner Group) showed different contaminants between Batch-1 360 and Batch-2 224 -- might the contaminants be important in cytotoxicity?
- Live-Dead assay is, however, limited:
  - Necessary to choose a single time point for analysis
  - Requires plasma membrane disruption and thus may not reflect HfO_2-induced cell compromise

Scrape Wound Assay to Evaluate Cytotoxicity

- Drag a glass pipet across an epithelial monolayer (16HBE14o- cells used for these studies)



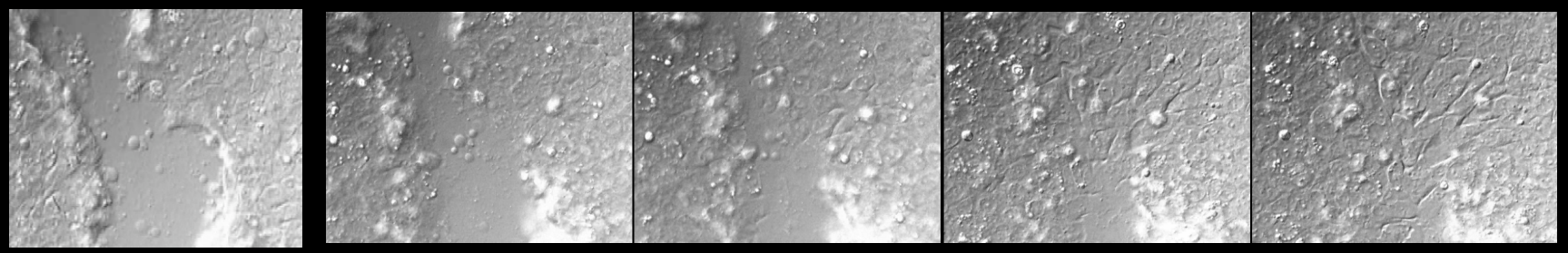


- Follow re-establishment of the monolayer using video microscopy

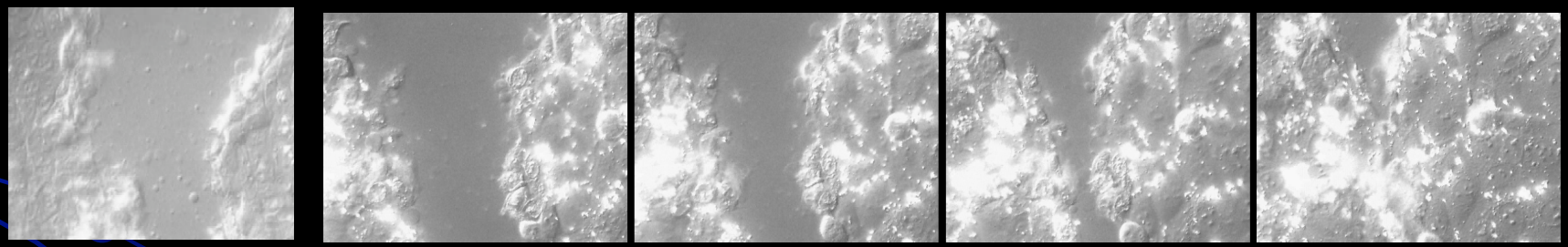
- We used this technique to evaluate if HfO_2 particles altered wound repair

Batch-1 360 HfO_2 Inhibits Wound Repair

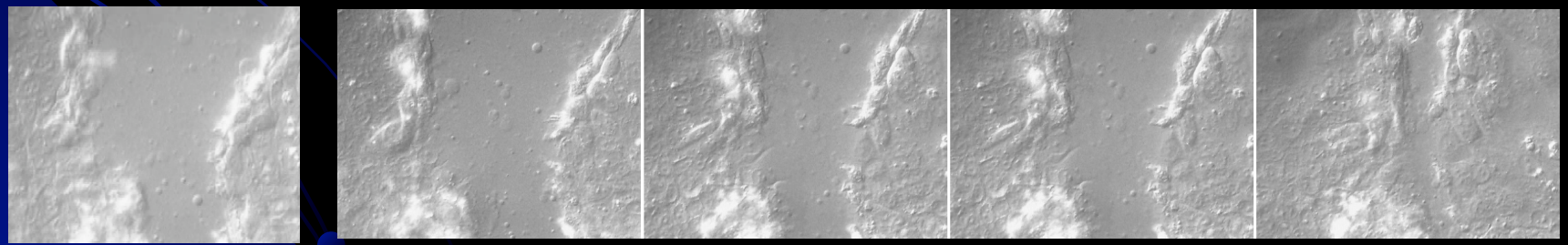
Note: MPG videos of each repair are available via download



250 ppm for 2 hr incubation and wash out for microscopy



50 ppm for 12 hr



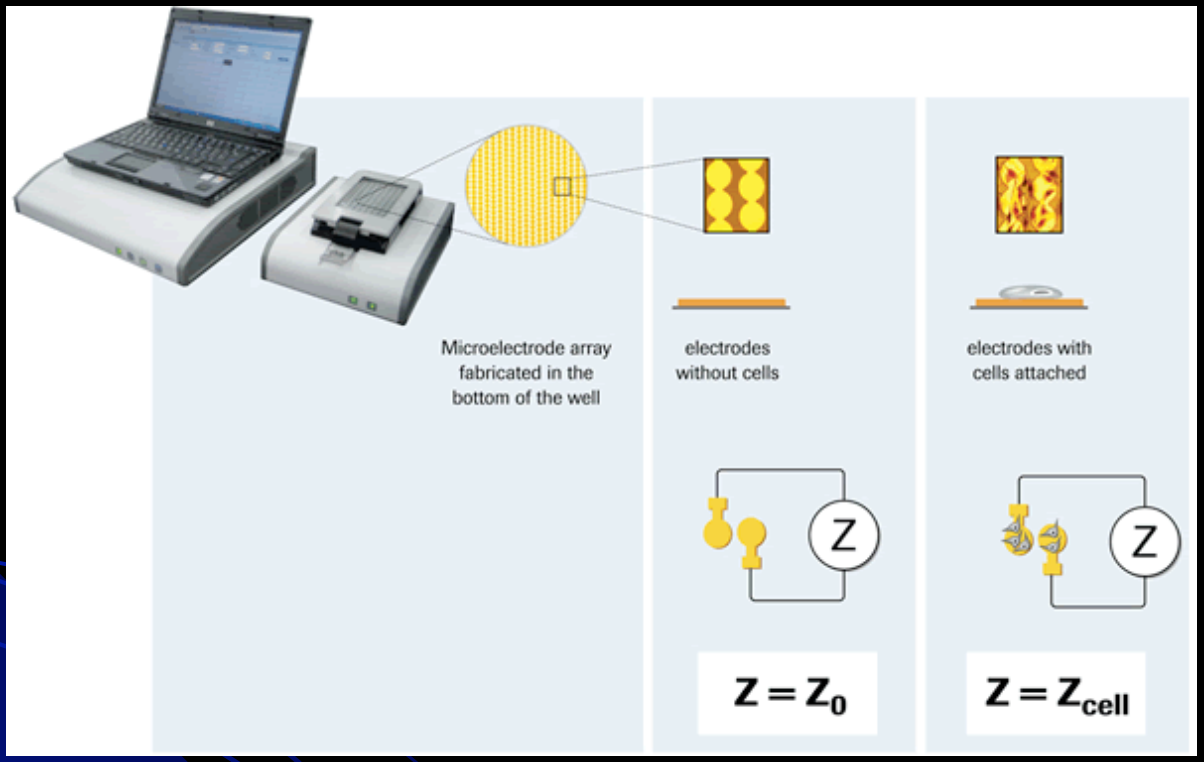
Scrape Wound Assay to Evaluate Cytotoxicity : Summary

- Scrape wound assay provides a sensitive measure of cellular physiology without the use of fluorescence
- HfO_2 has detrimental effects on cell spreading and/or migration (e.g., cytotoxicity) prior to inducing cell death
- Cellular effects of HfO_2 as low as 50 ppm (lower concentrations not yet tested)
- Results suggest that mechanisms involved in migration (e.g., adhesion) could be cytotoxic targets for HfO_2

- Scrape wound assay can be difficult to set up and, is limited for high throughput analysis

Measuring Cytotoxicity Using Impedance

- The xCELLigence System (Roche)

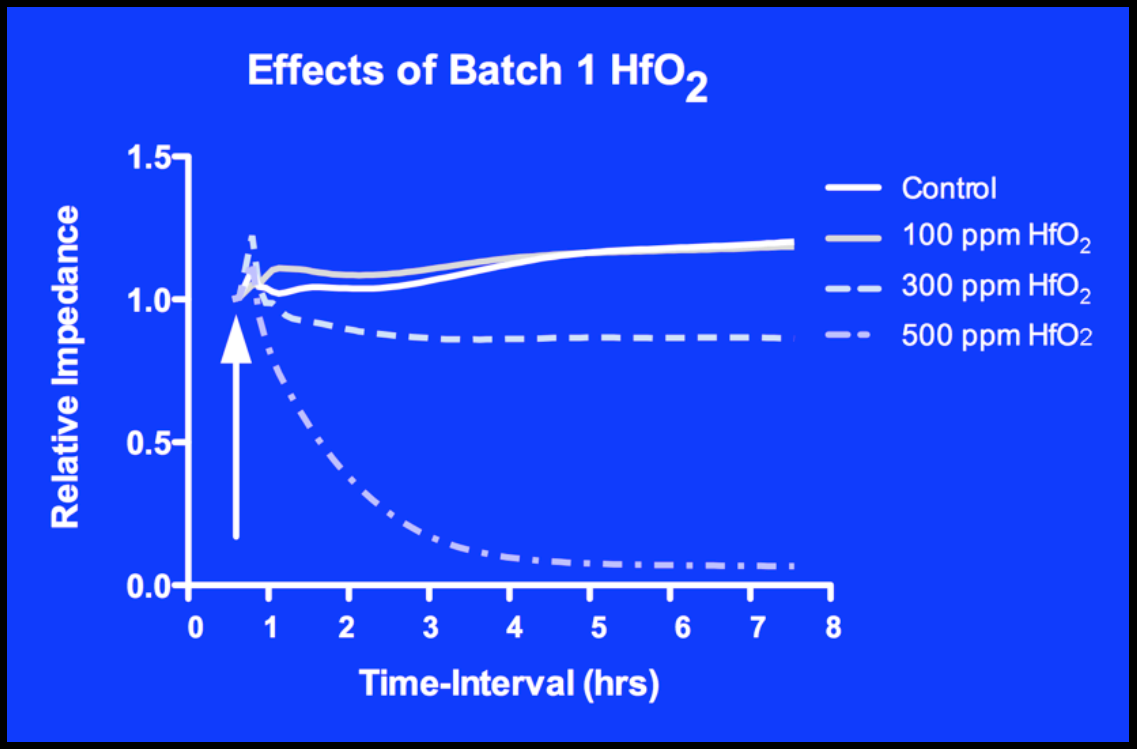


- Uses electrical impedance across interdigitated micro-electrodes integrated on the bottom of tissue culture E-Plates to monitor cellular events in real time without the incorporation of fluorescent labels

- In effect, more cells attached = more impedance
- As cells die (cytotoxicity), lose impedance
- 96 well format allows for relatively high throughput analysis

Impedance Measurement of Batch-1 360 Cytotoxicity on 16HBE14o- Cells

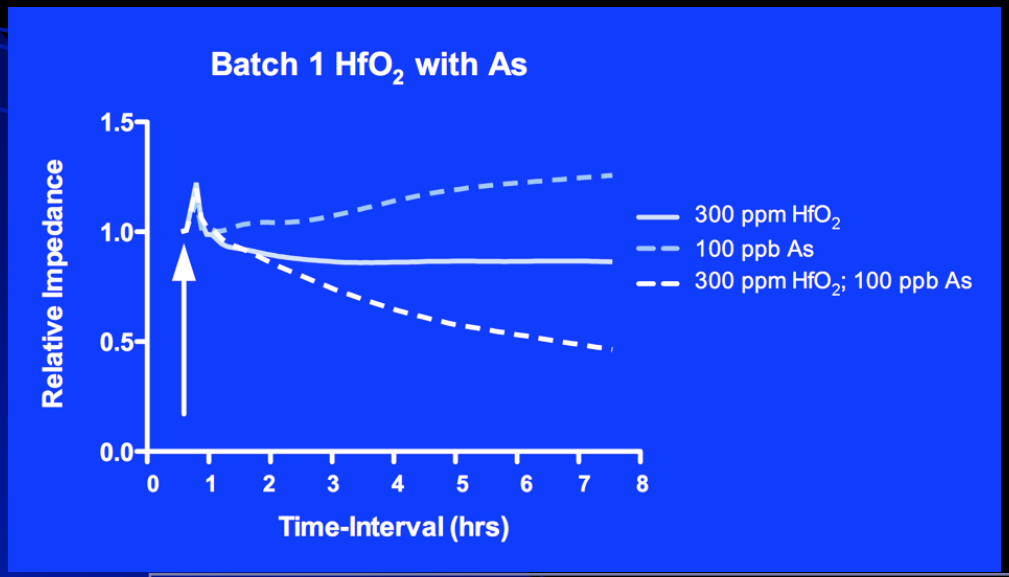
- Grow 16HBE14o- cells on E-Plates
- Add HfO_2 and monitor impedance over time
- Experiments done in series of n=6



- Cells tolerate 100 ppm HfO_2 over the 8 hr period
- 300 ppm and 500 ppm HfO_2 display increasing cytotoxicity
- Note agreement with Live-Dead assay at 2 hrs, but increased cytotoxicity of 500 ppm over time

Synergistic toxicity of HfO_2 and Arsenic

- Arsenic is a metalloid toxicant that is actively removed from regulated drinking water
- Iron nanoparticles are used to eliminate Arsenic from drinking water
- Arsenic can also be part of materials used in NP production
- Research Question: Does association with HfO_2 lead to cytotoxicity?



- 100 ppb As is not cytotoxic
- 300 ppm HfO_2 is slightly cytotoxic
- Pre-incubation of 300 ppm HfO_2 with 100 ppb Arsenic significantly increases cytotoxicity

Summary

- A mixture of assays can be used to evaluate cytotoxicity
- Size of HfO_2 alone cannot account for HfO_2 cytotoxicity (in agreement with Sierra Group Data)
- Contaminants and or HfO_2 shape/agglomeration can influence cytotoxicity -- how much each of these components contribute to cytotoxicity is unknown

- Considerations for future studies:
  - Immortalized cell models do not necessarily reflect *in vivo* tissue
  - Delivery of nanoparticles to cells not necessarily representative of *in vivo* environment

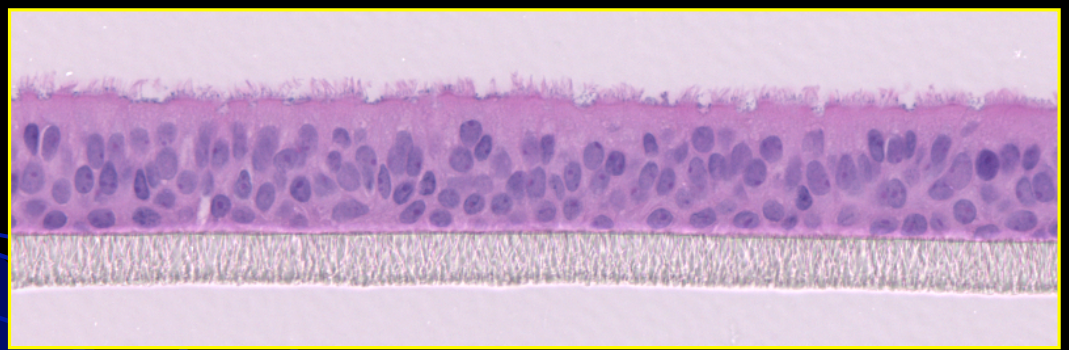
Future Directions

1) Improved characterization of model nanoparticles

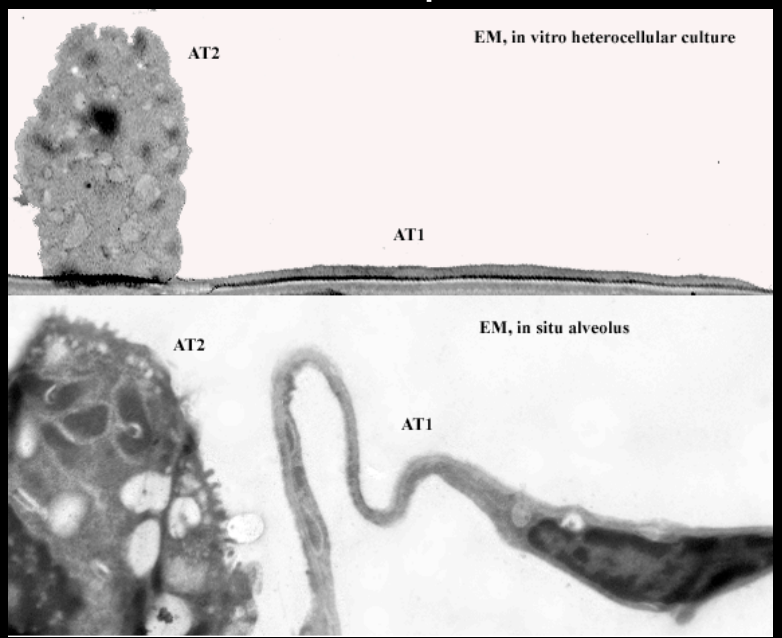
2) Understanding mechanisms of cytoxicity

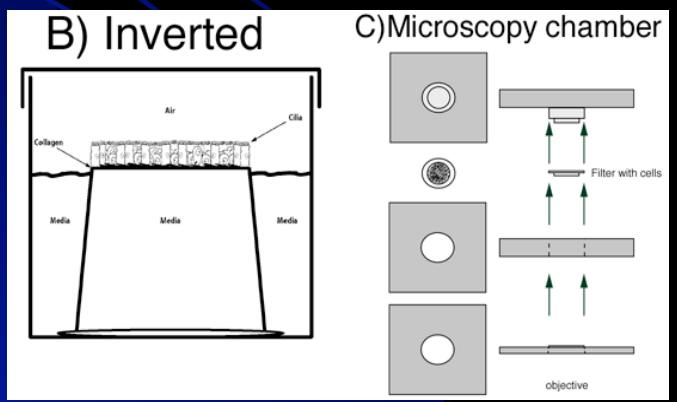
3) Improved cell models and delivery

Normal Human Bronchial Epithelial Cells



Rat Alveolar Epithelial Cells





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Credits

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