Assessing nanotoxicity at the single-cell level

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11.1 Need for single cell measures of nanotoxicity

11.1.1 There is more than one way for a cell to die...
11.1.2 "Necrosis" vs. "Apoptosis"
11.1.3 There are other forms of "toxicity"
11.1.4 Some other challenges in measuring toxicity of nanomaterials
11.2 Necrosis vs. Apoptosis mechanisms

11.2.1 Necrosis is unplanned "cell injury"

11.2.2 Apoptosis is planned "programmed cell death"

11.2.3 Why it is important to distinguish between necrosis and apoptosis?
Necrosis versus apoptosis


A famous paper from the literature...

Review Article

Cytometry in Cell Necrobiology: Analysis of Apoptosis and Accidental Cell Death (Necrosis)

Zbigniew Darzynkiewicz,* Gloria Juan, Xun Li, Wojciech Gorczyca, Tomoyuki Murakami, and Frank Traganos

The Cancer Research Institute, New York Medical College, Valhalla, New York

Received 16 May 1996; Accepted 22 July 1996
Morphological changes in cells undergoing apoptosis

See extensive and detailed figure legend in notes section of this slide.

Source: Darzynkiewicz et al 1997
FIG. 3. Schematic representation of apoptosis, oncosis, and necrosis, according to taxonomy of cell death proposed by Majno and Joris (67). The early stages of apoptosis are characterized by a relatively intact plasma membrane and intracellular changes as described in the legend to Figure 1 and in the text. During the late stage (apoptotic necrosis) the plasma membrane transport function fails resulting in cells that cannot exclude trypan blue or PI, and the remains of the apoptotic cell are engulfed by neighboring cells. During oncosis, cell mitochondria swell concomitant with a distortion of the mitochondrial structure and swelling of the whole cell. For some period of time, however, other vital cell functions are preserved albeit to different degrees. Rupture of the plasma membrane leads to a necrotic stage (oncotic necrosis) which is associated with local inflammation (modified, after Majno and Joris, ref. 67).
11.3 Single cell assays for necrosis and apoptosis

11.3.1 Dye exclusion assays for necrosis
11.3.2 TUNEL assays for late apoptosis
11.3.3 Annexin V assays for early apoptosis
11.3.4 COMET assays for DNA damage and repair
11.3.5 Light scatter assays
11.3.1  Dye exclusion assays for necrosis

- Cell membrane assay done on 8E5 cells

- (a) Control cells with no dye
- (b) Live cells stained with both PI and Hoechst dyes
- (c) Cells containing both dyes fixed in Cyto-Chex for two days
11.3.2 Single cell assays for early and late apoptosis

- DNA cleavage assay: TUNEL (late)
- Phosphatidylserine assay: Annexin V (early)
DNA Packaging: Nucleosomes and Chromatin

1. At the simplest level, chromatin is a double-stranded helical structure of DNA.

2. DNA is complexed with histones to form nucleosomes.

3. Each nucleosome consists of eight histone proteins around which the DNA wraps 1.65 times.

4. A chromatosome consists of a nucleosome plus the H1 histone.

5. The nucleosomes fold up to produce a 30-nm fiber...

6. ...that forms loops averaging 300 nm in length.

7. The 300-nm fibers are compressed and folded to produce a 250-nm-wide fiber.

8. Tight coiling of the 250-nm fiber produces the chromatid of a chromosome.

http://www.nature.com/scitable/topicpage/dna-packaging-nucleosomes-and-chromatin-310
From intranucleosomal cleavage to DNA ladders during apoptosis

Inter-nucleosomal cleavage results in a “DNA ladder” as DNA is broken down for reprocessing during late apoptosis.

http://www.nature.com/scitable/topicpage/dna-packaging-nucleosomes-and-chromatin-310

http://www.abcam.com/Apoptotic-DNA-Ladder-Isolation-Kit-ab65627.html
11.3.2 TUNEL Assay

- Late apoptosis assay
- TUNEL assay based on internucleosomal cleavage of nuclear DNA apoptotic cells into 200 base pairs in length.
TUNEL Assay by flow cytometry

APO-BrdU TUNEL Assay Diagram

DNA Strand Breaks caused by endonucleases produced by apoptosis process

Add BrdUTP’s to 3’-OH DNA Strand Breaks using TdT enzyme as catalyst

Fluorescentated antibody labelling of BrdUTP attached to 3’-OH DNA Strand Breaks

Courtesy:
Phoenix Flow Systems
http://www.phnxflow.com/apobrdu.html
Effectiveness of TUNEL

(a) Negative control of 8E5 cells
(b) Positive control of 8E5 cells with 100µM hydrogen peroxide

(a) Negative control, 8E5 cells without hydrogen peroxide
(b) Positive control, 8E5 cells that were incubated with hydrogen peroxide.

Leary lab data
Viability/Cytotoxicity of Ferric Oxide Nanoparticles by TUNEL Assay and flow cytometry

Cell density plots of Alexa 488 fluorescence versus forward light scatter for the following cell samples (A) negative control fixed lymphoma cell line, (B) positive control fixed lymphoma cell line, (C) post-fixed MOLT-4 cells exposed to 0.5 mg/mL nanoparticles, and (D) post-fixed MCF-7 cells exposed to 0.5 mg/mL nanoparticles. (Leary lab data)
11.3.3 Annexin V assays for early apoptosis

Uses the appearance of phosphatidylserine on the surface of apoptotic cells

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Flow cytometry results for Annexin V

- Problem:
  - Only two populations observed
  - PI still leaks out.
11.3.4 COMET Assay Verification of TUNEL Assays of Ferric Oxide Nanoparticle-Treated Cells

COMET assay performed on the following MOLT-4 cell samples: (a) untreated cells, (b) cells treated with 100µM hydrogen peroxide for 18 hours, (c) cells exposed to 0.1 mg/mL ferric oxide nanoparticles, and (d) cells exposed to 0.2 mg/mL ferric oxide nanoparticles.
11.3.5 Light scatter assays: MCF7 Cells Incubated with Different Concentration of Amino Group Quantum Dots® (QD)

- Amino-group functionalized QD are toxic to cells.
- The higher the concentration, the more toxic to cells
Uptake of Qtracker® (Qdots) by MCF7 cells at Different Incubation Times

Are they cytotoxic? Not by TUNEL, annexin V, or dye exclusion assays, but...
11.3.6 Dihydroethidium assays for oxidative stress
Qdot Cytotoxicity: Indications of oxidative stress and possible DNA damage by a more sensitive test

- Confocal imaging
  - ROS are present normally in cells. Heightened presence indicates a state of cellular stress.
  - Detection of ROS was observed in the positive control sample and the QTracker® sample.

<table>
<thead>
<tr>
<th>Dihydroethidium is shown in red QTracker® is shown in green.</th>
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<tbody>
<tr>
<td>(a) Control</td>
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<tr>
<td>(b) H₂O₂</td>
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<tr>
<td>(c) QTracker®</td>
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(a) Control                         (b) H₂O₂                         (c) QTracker®
11.4 Nanotoxicity in vivo – some additional challenges

11.4.1 Single cell nanotoxicity, plus....

11.4.2 Accumulations of nanoparticles can change toxicity locally to tissues and organs

11.4.3 Filtration issues of nanoparticles – size matters – toxicity to liver and lung
Some Challenges in Evaluating the "Toxicity" of Nanomaterials

- Toxicity of nanomaterials may be different from its elemental forms
- Toxicity may change with exposure to light, pH changes, etc.
- Toxicity is frequently masked by biocoatings which may be stripped at different rates by different cell types
- "Toxicity" needs to encompass assays beyond simple, rapid cell death, including: apoptosis, cell proliferation, cell differentiation, changes in cell function, etc.
- How do we evaluate multi-component nano "platform technologies, e.g. nanodelivery systems so they can be re-used."
References