NIST at ICCVAM: Tools to improve confidence in alternative test methods

Elijah Petersen and John Elliott
Cell Systems Science Group
Material Measurement Laboratory
National Institute of Standards and Technology
Some key focuses at NIST

- **Measurements**
  - Develop new measurement methods
  - Improve accuracy/precision of measurements

- **Reference Materials**
  - Well-defined materials for use as a reference when making measurements
  - Enables inter-lab comparability
  - Physical artifacts for calibrating instruments

- **Standards**
  - Documentary standards, ASTM, ISO
  - Reference data (chemical spectra)
  - Technical Notes: “Guidelines for Evaluating and Expressing the Uncertainty of NIST Measurement Results” (GUM)

- **Biology/biotechnology**
  - Cell-related measurements and technology (~1990)
  - Cytotox measurements, organism measurements (~2005)

- **Food-matrix reference materials to facilitate nutritional labeling**

- **NIST Synthetic RNA controls (ERCCs) used in sequencing of Ebola virus genomes to characterize patterns of viral transmission**
Cause and Effect Analysis: A new approach for developing robust nano-bio assays
Workshop hosted by EMPA (Switzerland) on June 18 & 19, 2015
16 participants in attendance from 3 countries

Evaluated five *in vitro* assays for use with nanoparticles:
MTS assay (cell viability)
DCF-DH assay (ROS generation)
Flow cytometry assay (quantification of viable, necrotic, or apoptotic cells)
Comet assay (genotoxicity)
ELISA assay for IL-8 (inflammation response)

For each assay, we developed a flow chart, cause-and-effect analysis, and control experiments
Flow charting

**MTS**
- Seed cells in 24 well plate
- Incubate for 24 h
- Remove the medium
- Treat with nanoparticles and chemical control
- Incubate for 3, 24h, 72h
- Cell harvest
- Staining
- Flow cytometric analysis
- Fluorescence measurements after 1, 2, 3 and 4 hours

**DCF-DH**
- Seed cells in 24 well plate
- Washing with PBS (2 x)
- Cell staining with membrane permeable H2DCF-DA
- 2 washing steps (PBS)
- Remove the medium
- Treat with nanoparticles and chemical control

**ELISA**
- Seed cells in 24 well plate
- Washing with PBS (2 x)
- Cell staining with membrane permeable H2DCF-DA
- 2 washing steps (PBS)
- Remove the medium
- Treat with nanoparticles and chemical control
- Cell harvest
- Harvest supernatant
- Wash high binding 96 well plate with 100 μL specific capture antibody
- 5 washing steps
- Block nonspecific binding with 200 μL 3% casein
- 5 washing steps
- Apply 100 μL specific antibody
- 5 washing steps
- Apply 100 μL secondary HRP
- 7 washing steps
- Apply 100 μL substrate (TMB)
- Measure OD at 450 nm

**Flow**
- Seed cells in 24 well plate
- Washing with PBS (2 x)
- Cell staining with membrane permeable H2DCF-DA
- 2 washing steps (PBS)
- Remove the medium
- Treat with nanoparticles and chemical control
- Cell harvest
- Pellet binding
- Sample preparation
- Alkaline cell lysis
- DNA unwinding
- Electrophoresis
- Neutralization
- Deproteinization
- Drying
- Ethidium bromide staining
- Microscopic analysis
- Absorbance measurements at 450 nm

**Comet assay**
- Seed cells in 24 well plate
- Washing with PBS (2 x)
- Cell staining with membrane permeable H2DCF-DA
- 2 washing steps (PBS)
- Remove the medium
- Treat with nanoparticles and chemical control
- Cell harvest
- Remove supernatant
- Insoluble with MTS containing medium

**Cell seeding/pretreatment**

**Nanoparticle treatment**

**After nanoparticle exposure**

**Instrument analysis**
MTS cell viability assay

Summary Instructions:

1. Receive NP, serum, cells, chemical control
2. Negative control- no treatment
3. Positive control- 100 uM CdCl2
4. Manufacturer’s protocol
5. Cell proliferation rate- 21h
6. Normalize treatment to no-treatment well
Identify sources of variability using cause & effect analysis

What is the purpose of cause and effect analysis?

1. Method to lay out implicit knowledge

2. Systematic approach to identify potential sources of variability in an assay and to highlight key sources of variability

3. Can be used to help design process control experiments, improve plate layout, and with writing a protocol

4. Can be used iteratively to improve assay quality by decreasing variability in key assay steps which decreases the total variability in the assay
Design a new plate format with process control measurements

- Control 1 - within-pipette volume, reagent rinsing and chemical interference control
- Control 2 - positive chemical toxin response control (triplicate)
- Control 3 - within-pipette cell seeding density, general rinsing and cell growth control
- Control 4 - within-pipette volume, general reagent quality and background measurement control
- Control 5 - NP interference control
- Control 6 - Instrument control (optional)
- Control 7 - between-pipette variability (chemical ctrl vehicle)
- Control 8 - between-pipette variability (NP test vehicle)

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Dosage concentration (units are μg/mL for NPs and μmol/L for CdSO₄)

- 0
- 1
- 10
- 25
- 50
- 100

Plate design includes 8 control measurements in addition to the NP measurement
Cell pipetting caused highest amount of variability among controls.
Interlaboratory comparison with MTS assay

- 5 national metrology institutes were involved in the interlaboratory comparison
- Experimental design:
  - Share two A549 cell lines from ATCC and EMPA
  - Serum from local provider
  - Reagents from local provider
  - Serum and serum-free tests
  - Multiple replicates
  - Share nanoparticles (+ve PS) and chemical control (CdCl₂)
- Looks like harmonization between the laboratories
- No cell line differences
- The serum conditions increases variability
Can the system control measurements identify the cause of the outlier?

- Chemical Process Control- tests overall measurement system

Serum free conditions, variability less than with NP

Differences between cell lines
How sensitive are we to cell seeding variability

- Correlation between no-treatment cells and NP EC50
- If outliers are removed, no strong correlation
- Suggests that within this range of cell seeding variability (OD=1.5-2.5) no big effect on EC50
Impact of cell rinsing for lab A

Changing the rinsing procedure brought lab A results to the interlab consensus values.
What is the purpose of process control measurements?

1. Provide evidence that the measurement process occurred as expected.

2. Should meet specifications before acceptance of the test result.

3. Can be used to identify relative contributions to total variability in assay result. Protocol modifications?

4. Ideal for designing protocols for an interlaboratory comparison

5. Can be used to assess the functioning of different components in a complex assay
Instrument calibration: Process for Determining Analytical Performance of a Widefield Fluorescence Microscope

Series of image pairs acquired over a range of exposure times (example)

The difference between images is used to compute pixel variance (‘noise’)

- Inspired by Photon Transfer Curve:

- Saturation = \( \arg \max_{t>0} (\sigma_{glass}^2(t)) \)

Instrument calibration: Establishing Instrument Specifications

By *charting* the Detection Threshold, Saturation, and Intensity Response* over time, you can:

- Demonstrate comparability between fluorescence intensity measurements
- Identify changes in the analytical performance of your widefield microscope

*can be used to normalize for day-to-day intensity variations
Potential NIST-ICCVAM interactions

• Current- Protocol evaluation for electrophilic allergen screen assay
  – Cause-and-effect analysis
  – Process control considerations
  – Discussion
  – Potential involvement in interlaboratory comparison
Collaborators

NIST Scientists
Michael Halter
Alex Tona
Elijah Petersen
John Elliott
Blaza Toman

External Collaborators
Matthias Rosslein (EMPA)
Harald Krug (EMPA)
Peter Wick (EMPA)
Cordula Hirst (EMPA)
Rawiwan Maniratanochote (NANOTEC)
Nam Woong Song (KRISS)
Francois Rossi (JRC)
Agnieska Kinsner-Ovaskainen (JRC)