Advances in the reliability of nanotoxicity assays

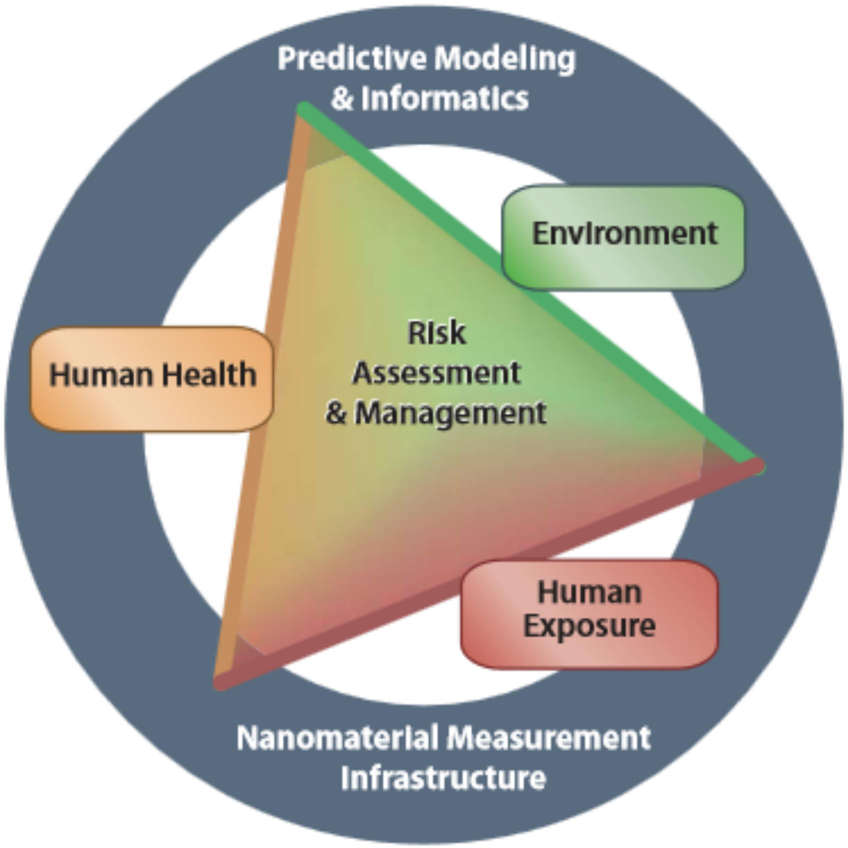
Elijah Petersen

National Institute of Standards and Technology

NIST
National Institute of Standards and Technology
U.S. Department of Commerce

MATERIAL
MEASUREMENT
LABORATORY

NIST ROLE IN NANOTECHNOLOGY ENVIRONMENTAL HEALTH AND SAFETY (NANOEHS)



National Nanotechnology Initiative 2011 Environmental Health and Safety Research Strategy

NIST Reference Nanomaterials

Gold nanoparticles (10, 30, and 60 nm)
Single-wall carbon nanotube (raw soot) and dispersed into three length populations
Titanium dioxide nanoparticles (made from Degussa P25)
2 nm silicon nanoparticles
Silver nanoparticles (75 nm, 10 nm in preparation)
Multiwall carbon nanotube (raw soot)

Can be useful for interlaboratory comparisons, instrument validation and calibration, and positive and negative controls for nanotoxicity studies

Critical for establishing comparability of nano-related measurements.

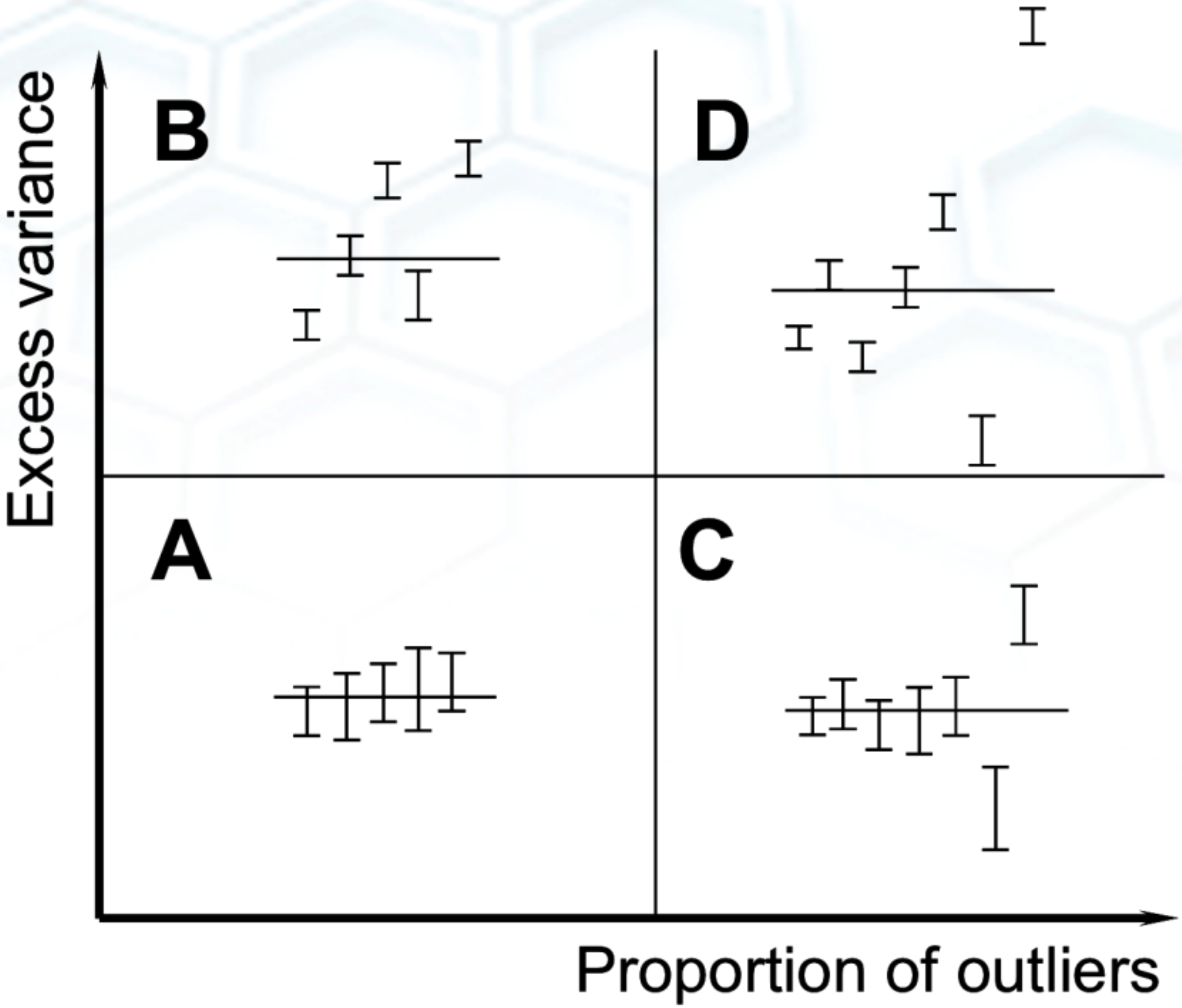
Vincent Hackley

Documentary standards

NIST participates in standards organizations that provide validated documentary standards on a range of topics

- Nanoparticle characterization using a range of instruments for all nanoparticles (DLS, TEM, etc.) through the NIST/NCL protocols
- Sonication protocols that provide reproducible, traceable NP sonication between instruments and laboratories
- MTS assay for cell toxicity from nanomaterials
- Guidance document for aquatic toxicity testing of nanomaterials

DESIGN FOR COMPARABLE DATA



Data indicate means and error bars

"CCQM Guidance Note: Estimation of a consensus KCRV and associated Degrees of Equivalence" Draft 2010-03-01, Stephen LR Ellison, LGC and Maurice Cox, NPL

ARTIFACTS IN NANOECOTOXICOLOGY MEASUREMENTS

Environmental Science & Technology
Critical Review
pubs.acs.org/est

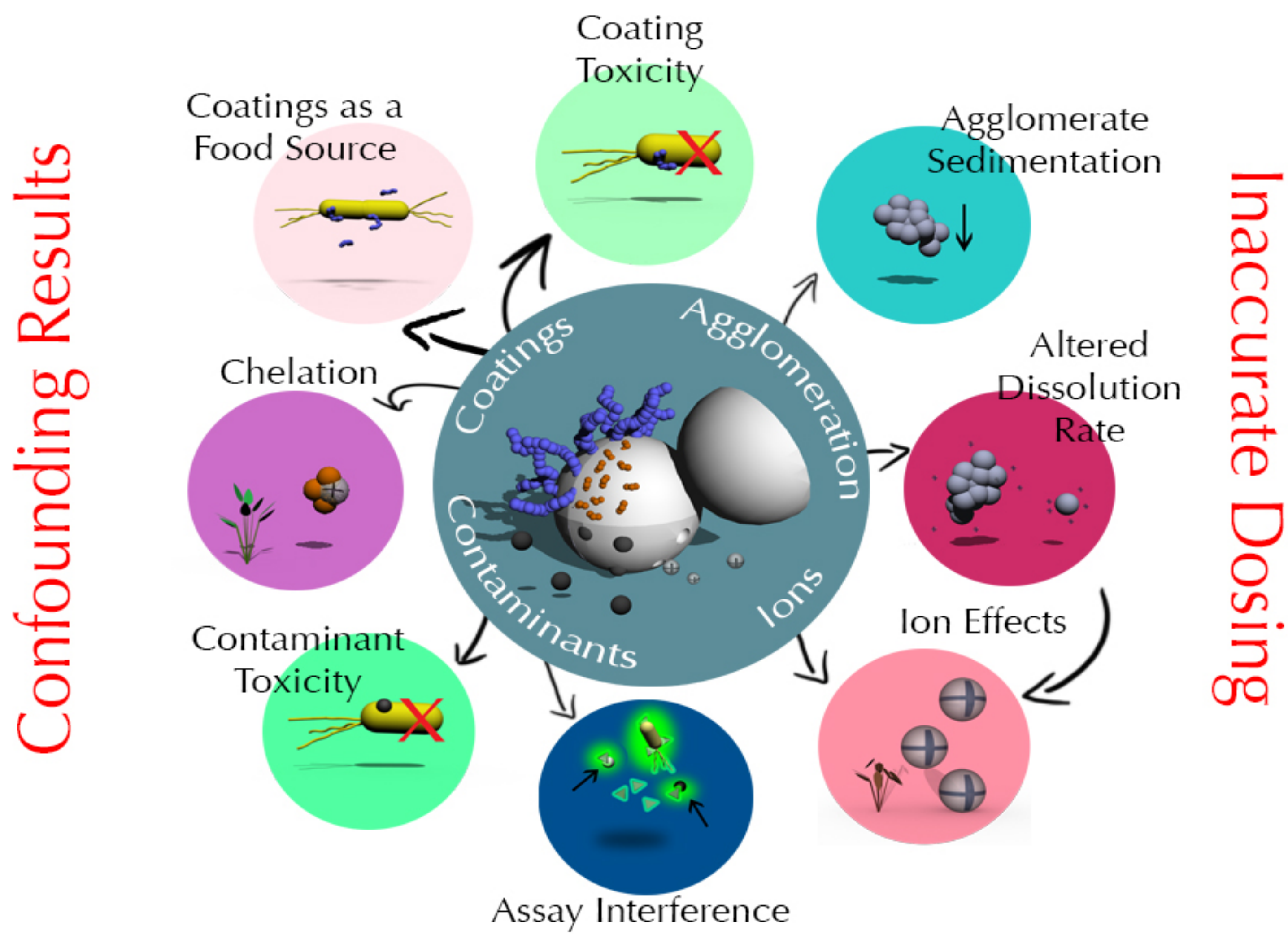
Identification and Avoidance of Potential Artifacts and Misinterpretations in Nanomaterial Ecotoxicity Measurements

Elijah J. Petersen,[†,*] Theodore B. Henry,[‡,§,∥] Jian Zhao,[⊥] Robert I. MacCuspie,[#,∇] Teresa L. Kirschling,[○] Marina A. Dobrovolskaia,[◆] Vincent Hackley,[#] Baoshan Xing,[⊥] and Jason C. White[¶]

Artifacts can potentially occur at each step of nanoecotoxicology testing

1. Procurement of NPs (impurities, incorrect sizes)
2. Storage (dissolution, release of coatings)
3. Dispersion (ROS from ultrasonication)
4. Measurement of toxic endpoints (interaction with test reagents)
5. Characterization in tissues (misidentification using TEM)

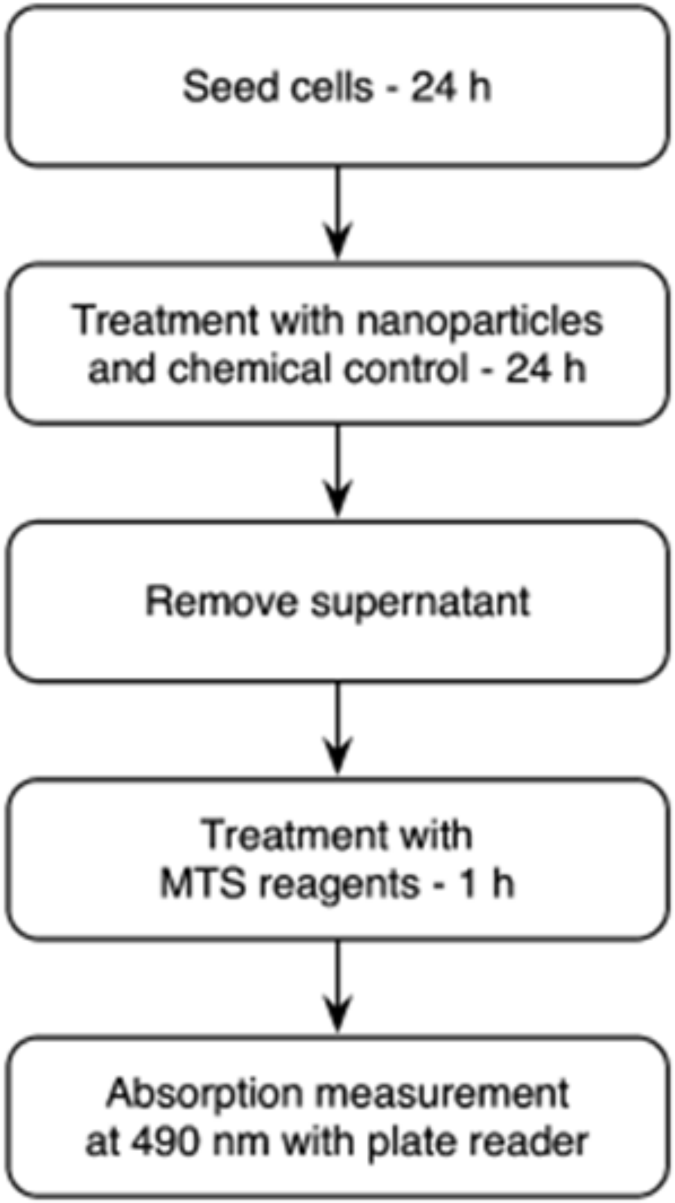
NANOECOTOXICOLOGY ARTIFACTS



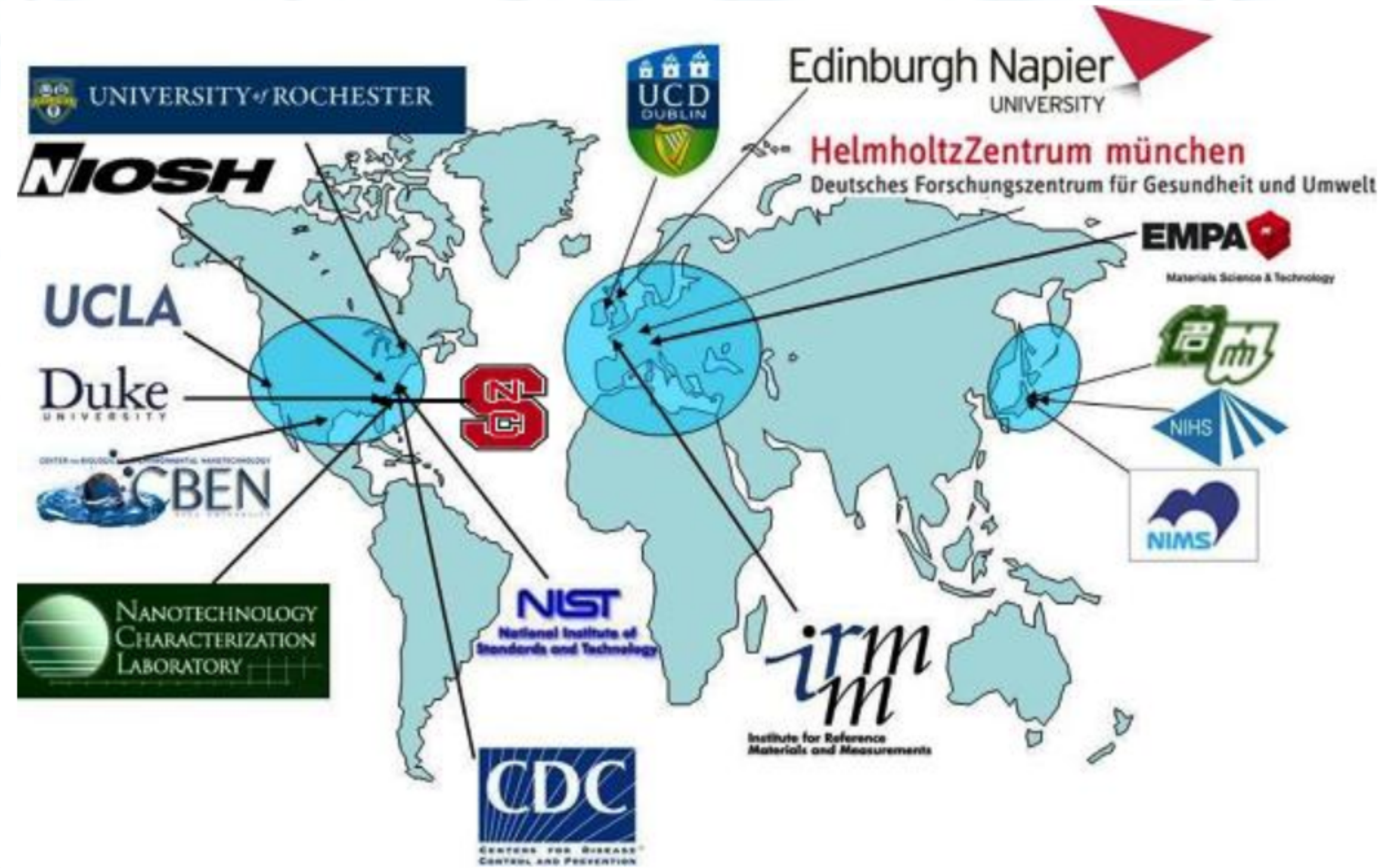
Petersen et al., 2014, Environ. Sci. Technol. 48(8), p 4226-4246.

DEVELOPMENT AND INTERLABORATORY TESTING OF MTS NANOCYTOTOXICITY ASSAY

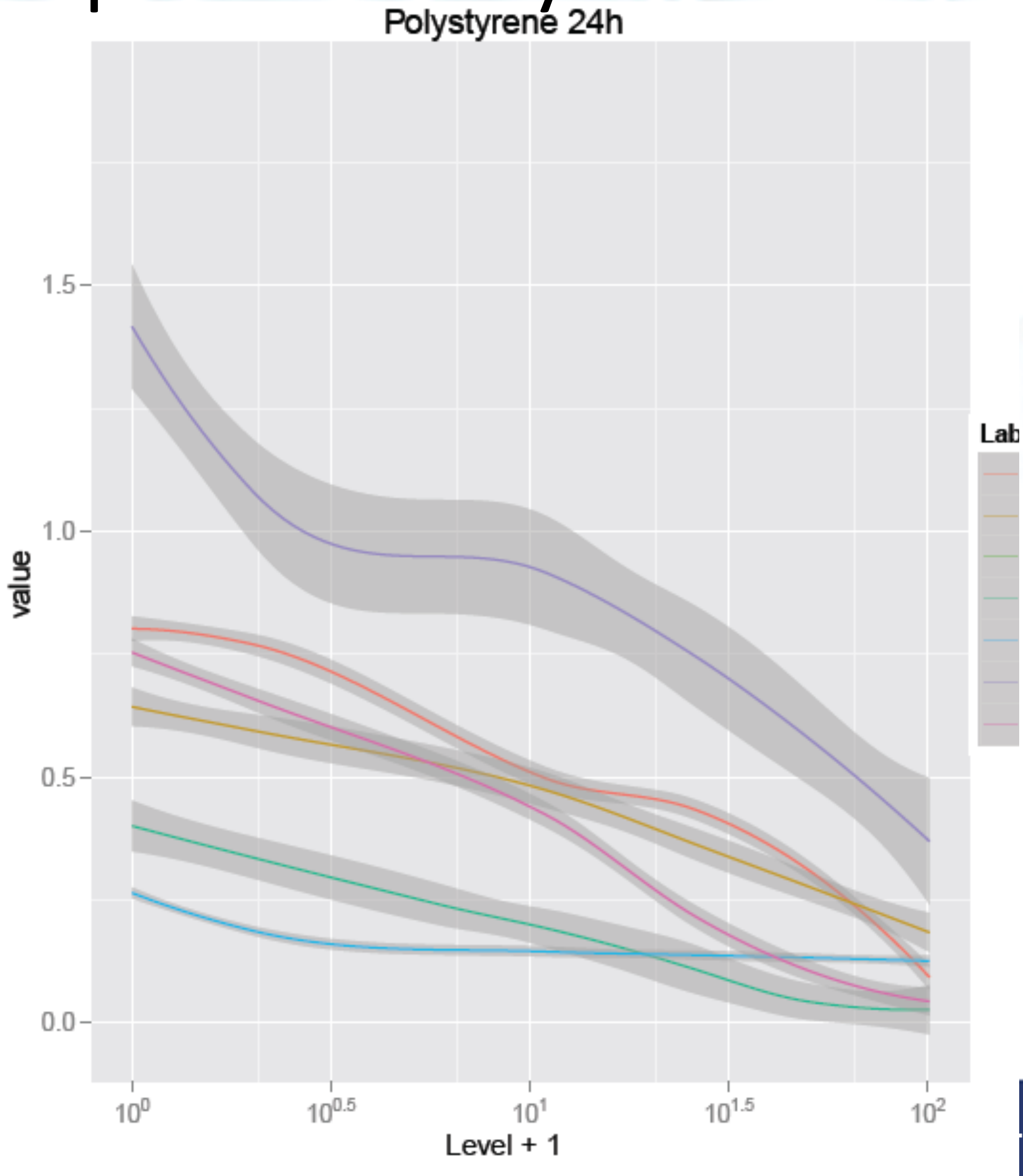
Flowchart with the main process steps of the MTS Assay



IANH International Alliance for NanoEHS Harmonization



Raw Data-absolute absorbance, individual scale, all experiments by different labs



- Variations in absolute absorbance.
- Variations in response shape.
- All do show a "toxic" trend.

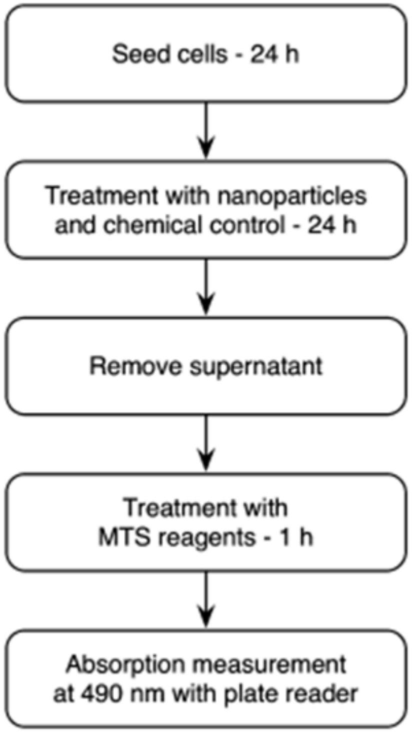
WHAT CAN WE DO TO INCREASE CONFIDENCE IN THE MEASUREMENT

Treat the assay as a measurement process

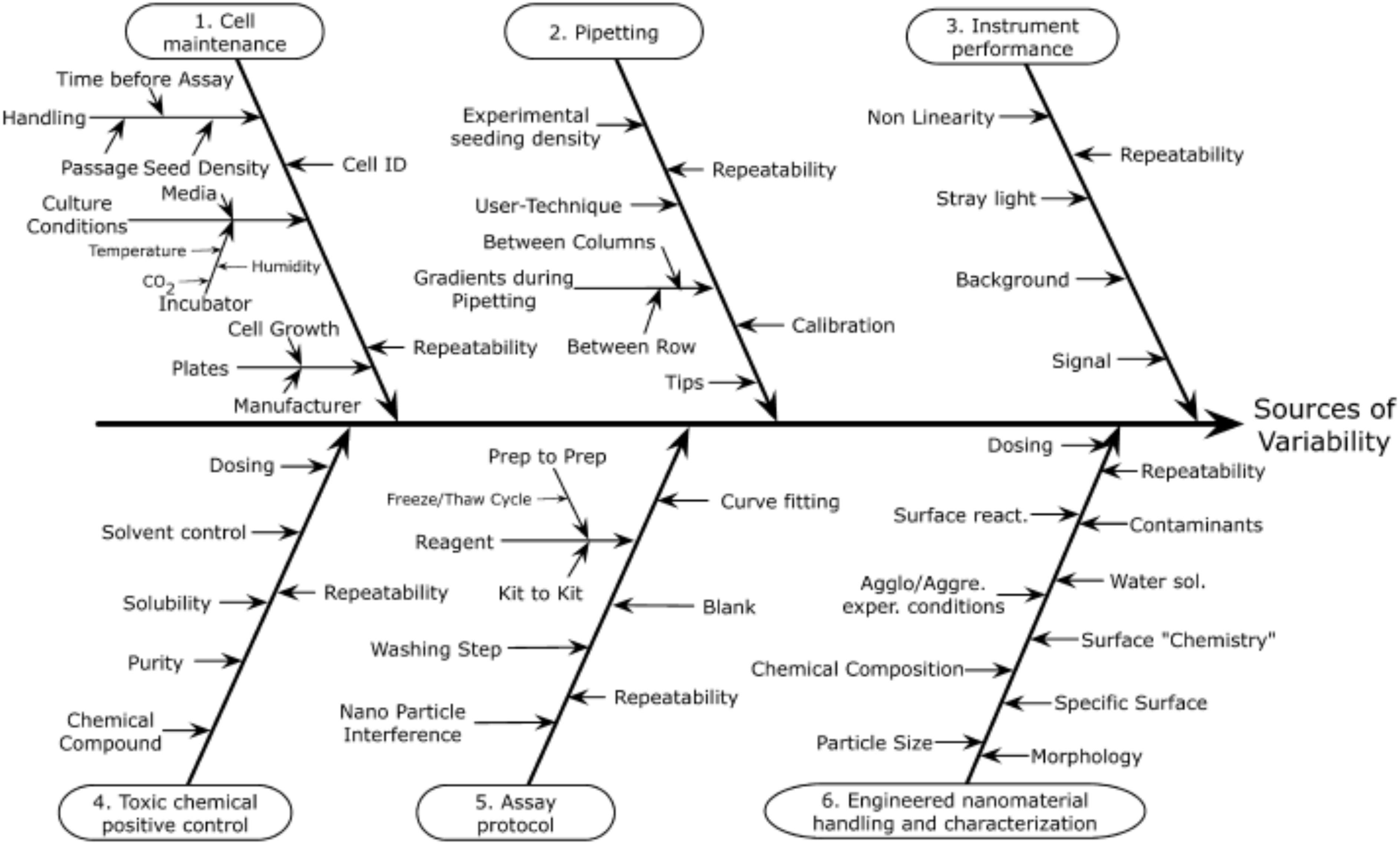
Add process controls as evidence that the measurement process is proceeding as expected

Adapt the “seven basic tools for quality” to cell assays

- Cause and effect diagram
- Check sheet
- Control charts
- Histogram
- Pareto chart
- Scatter diagram
- Flow chart

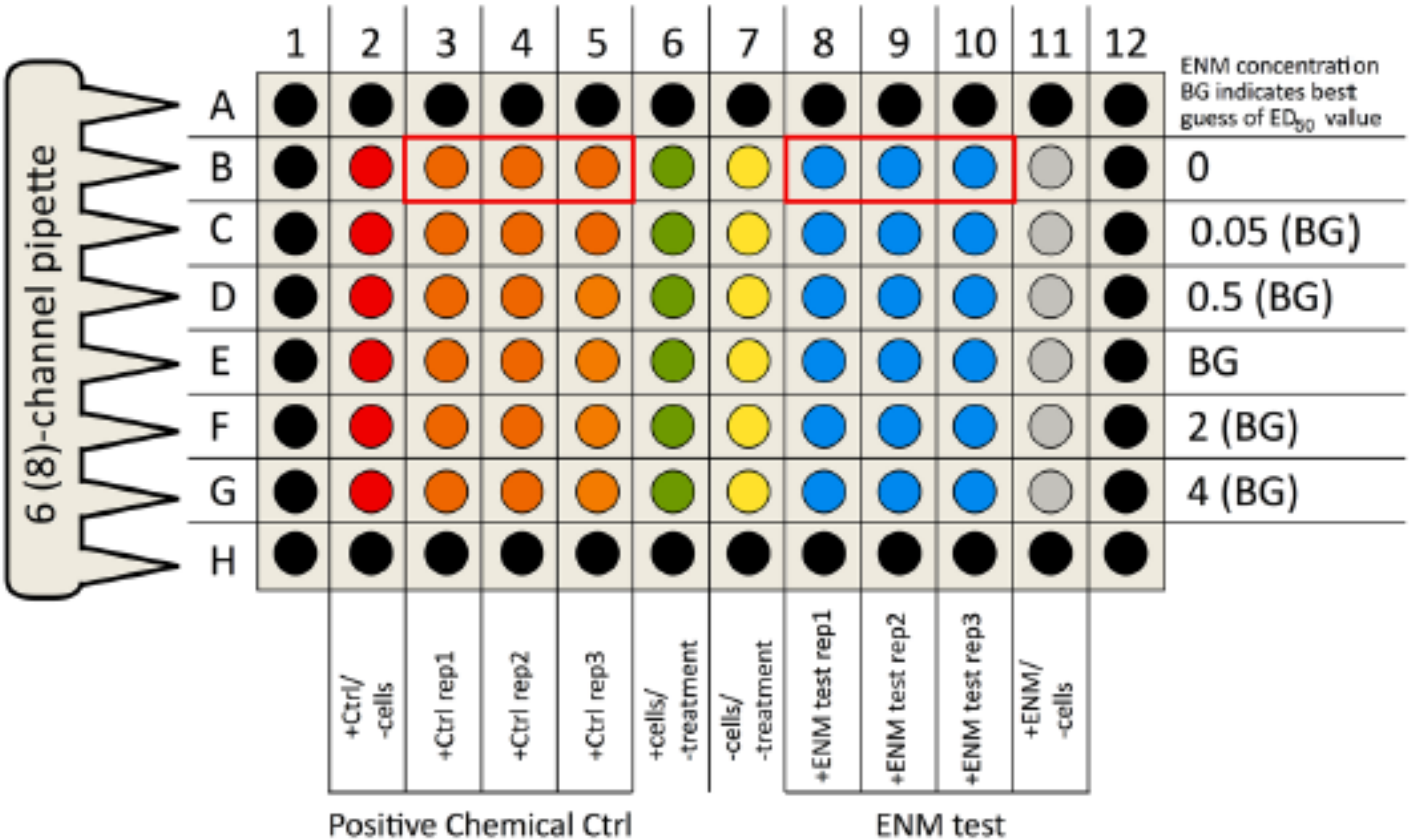


Cause & Effect Analysis of MTS Assay



Roesslein et al., 2014, Chemical Research in Toxicology, 2015, 28 (1), 21-30

Novel 96 well plate layout with control experiments



INTERLABORATORY COMPARISON

- 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 ($CdCl_2$)

KRISS Korea Research Institute of Standards and Science

EMPA Materials Science & Technology

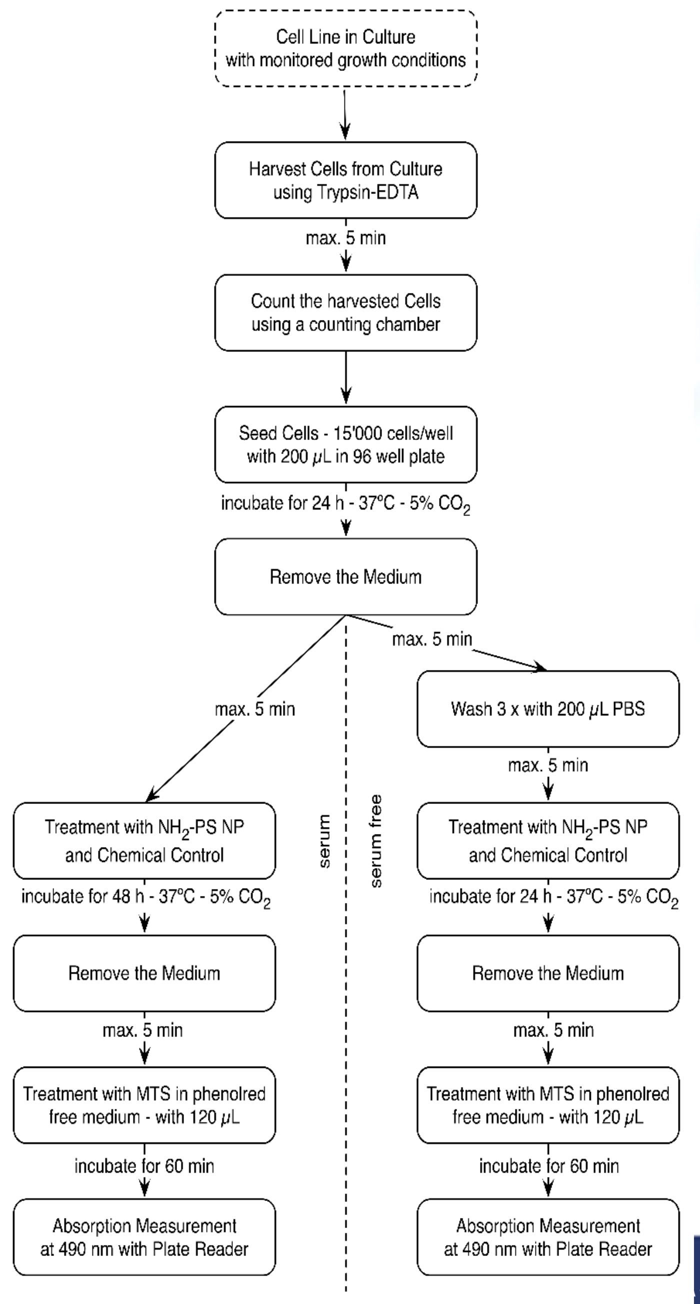
NIST

European Commission JOINT RESEARCH CENTRE The European Commission's in-house science service

10th anniversary NANOTEC a member of NSTDA

Elliott et al., 2017, Altex, 34, 201-218.

Flowchart with the main process steps of the MTS Assay

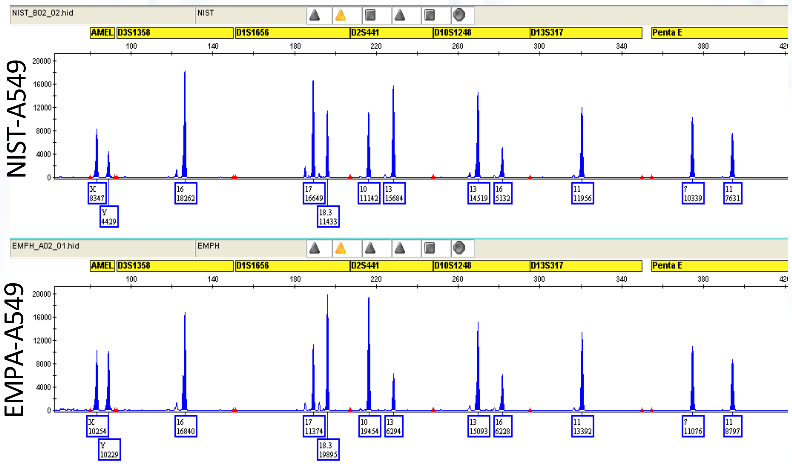


Two cell lines were tested in the interlab comparison

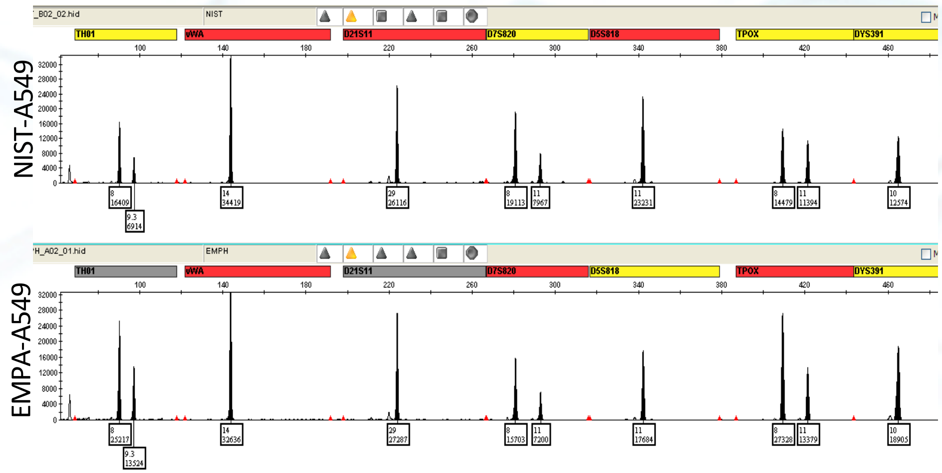
Cell line	Cell cycle time (h)	Medium volume (um^3)[1]	Short Tandem Repeat (STR) analysis[2]
A549-A	22.5±2.4	2047±90	In agreement with ATCC
A549-B	22.6±2.2	2327±94	Missing allele 12 (CSF1PO)

STR analysis of the two cell lines

a. FAM dye channel

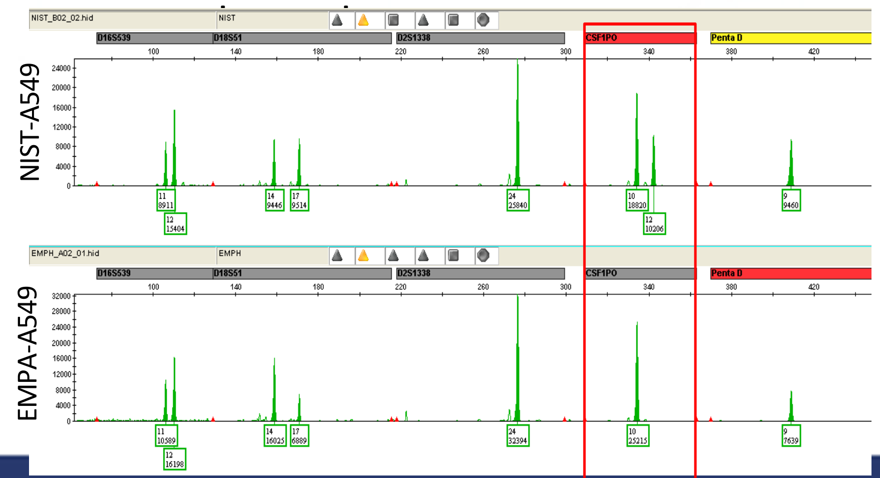


b. NED dye channel

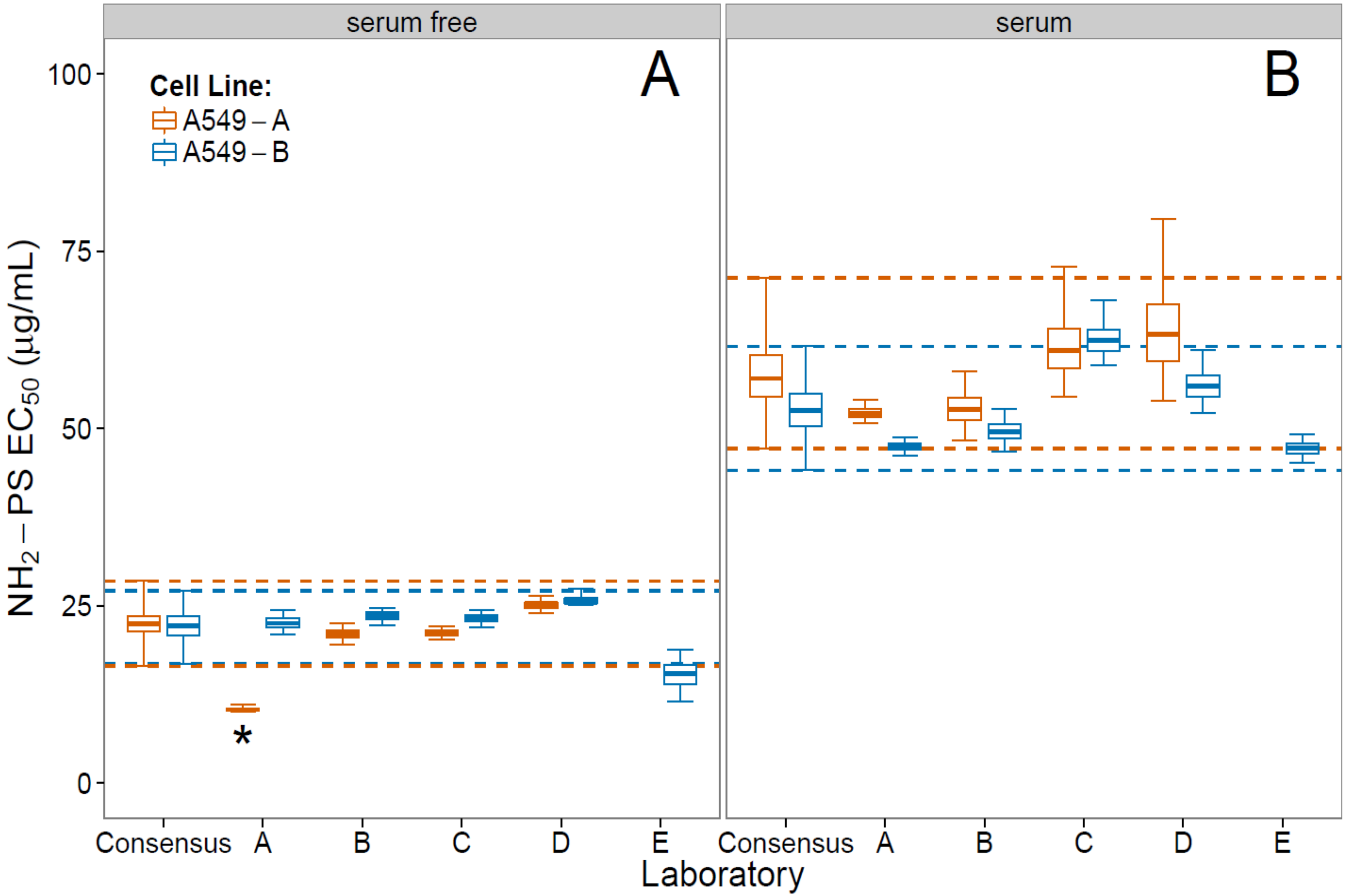


c. PET dye channel

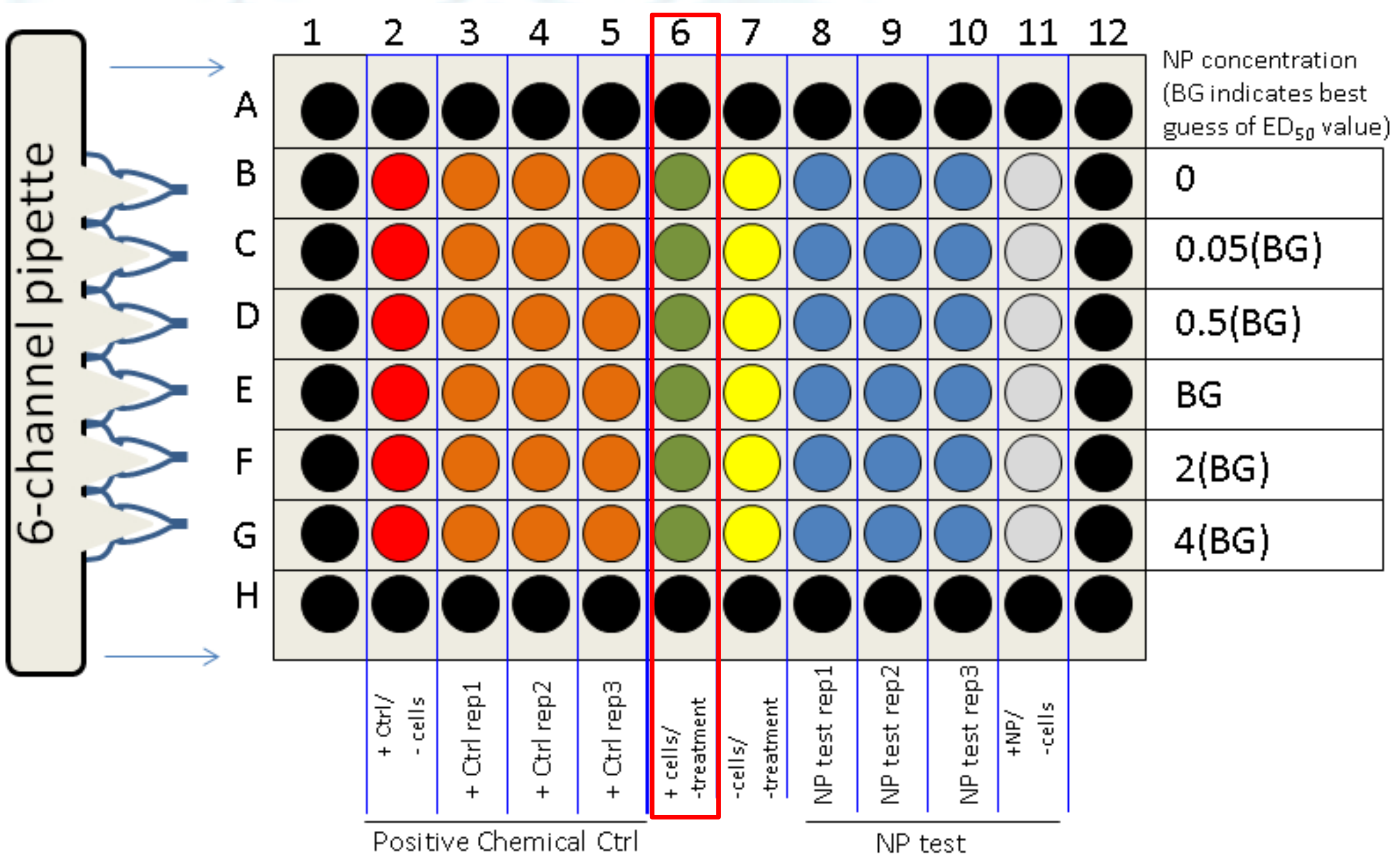
d. VIC dye



Interlaboratory Agreement with Positively Charged Polystyrene Nanoparticles

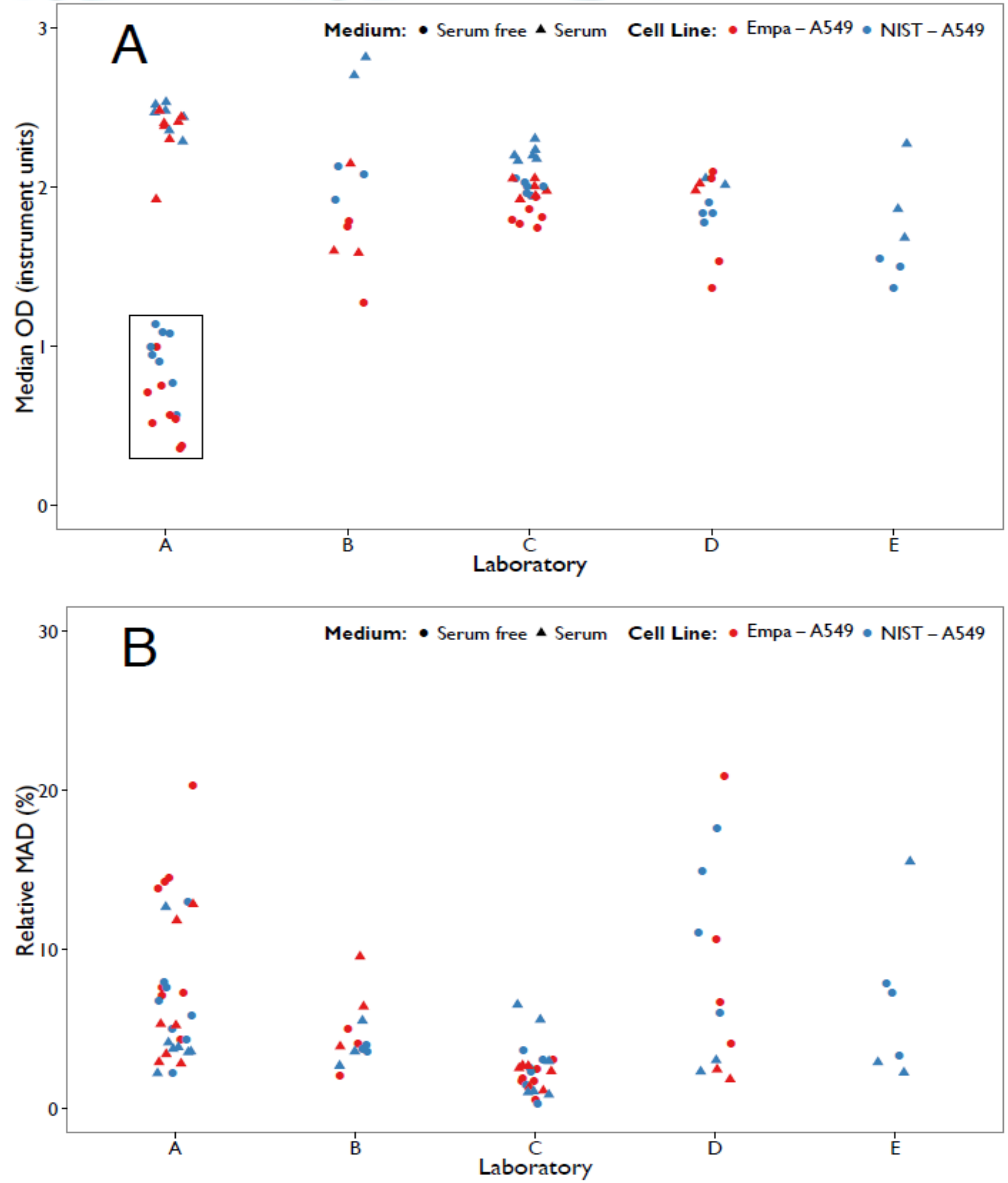


Design Element 1: Within Multichannel Pipette (MCP) Seeding Density

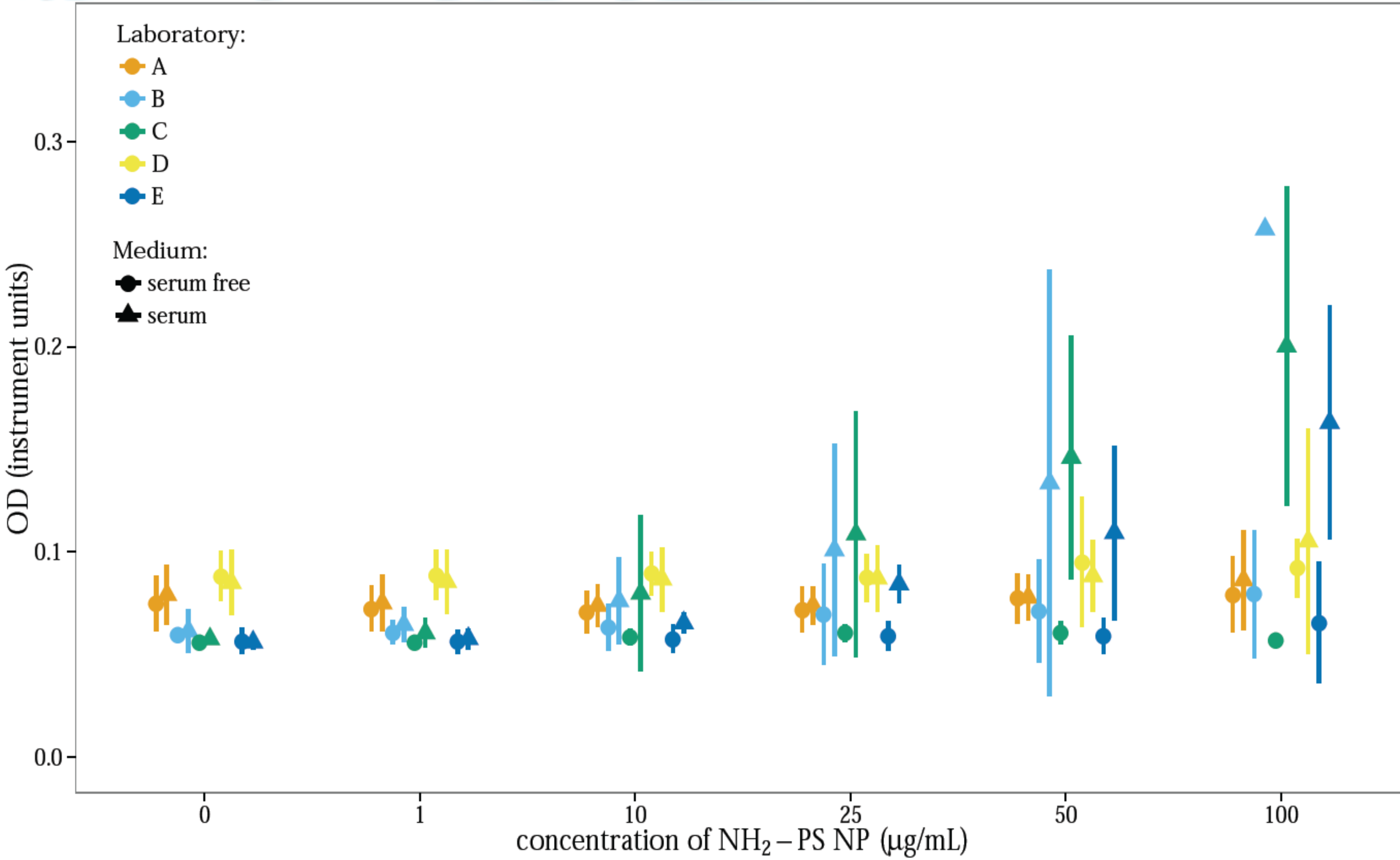


Assesses **within** multichannel pipetting variance. Non-treated cells seeded with a single multichannel pipette ejection step. Absolute absorbance measurement provides insight on nominal cell growth. Indicates technical problems with the pipette.

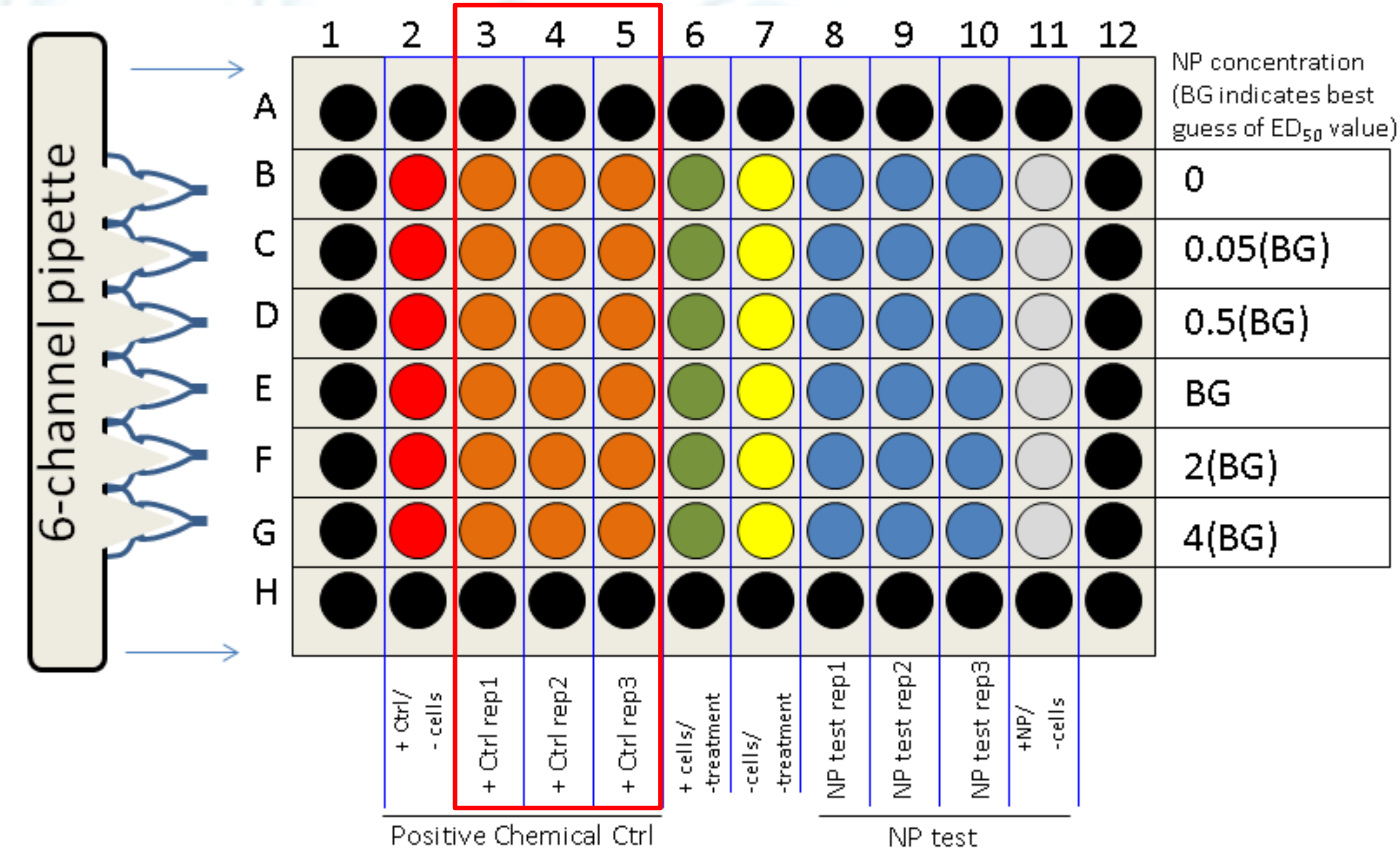
Design Element 1: Within Multichannel Pipette (MCP) Seeding Density



Design Element 4: Nanoparticle influence on assay readout (after rinsing)

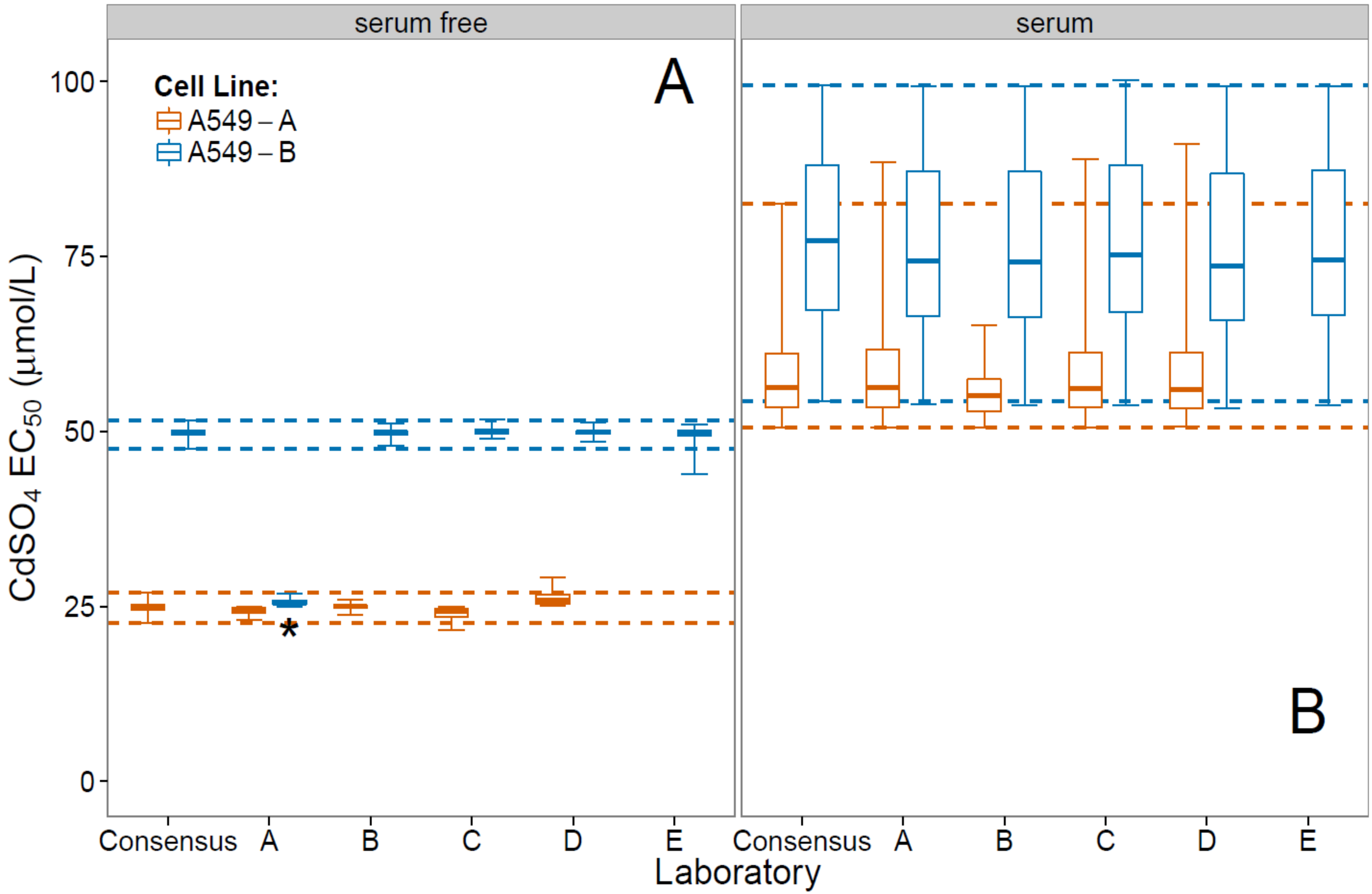


Design Element 5: Chemical Control dose response

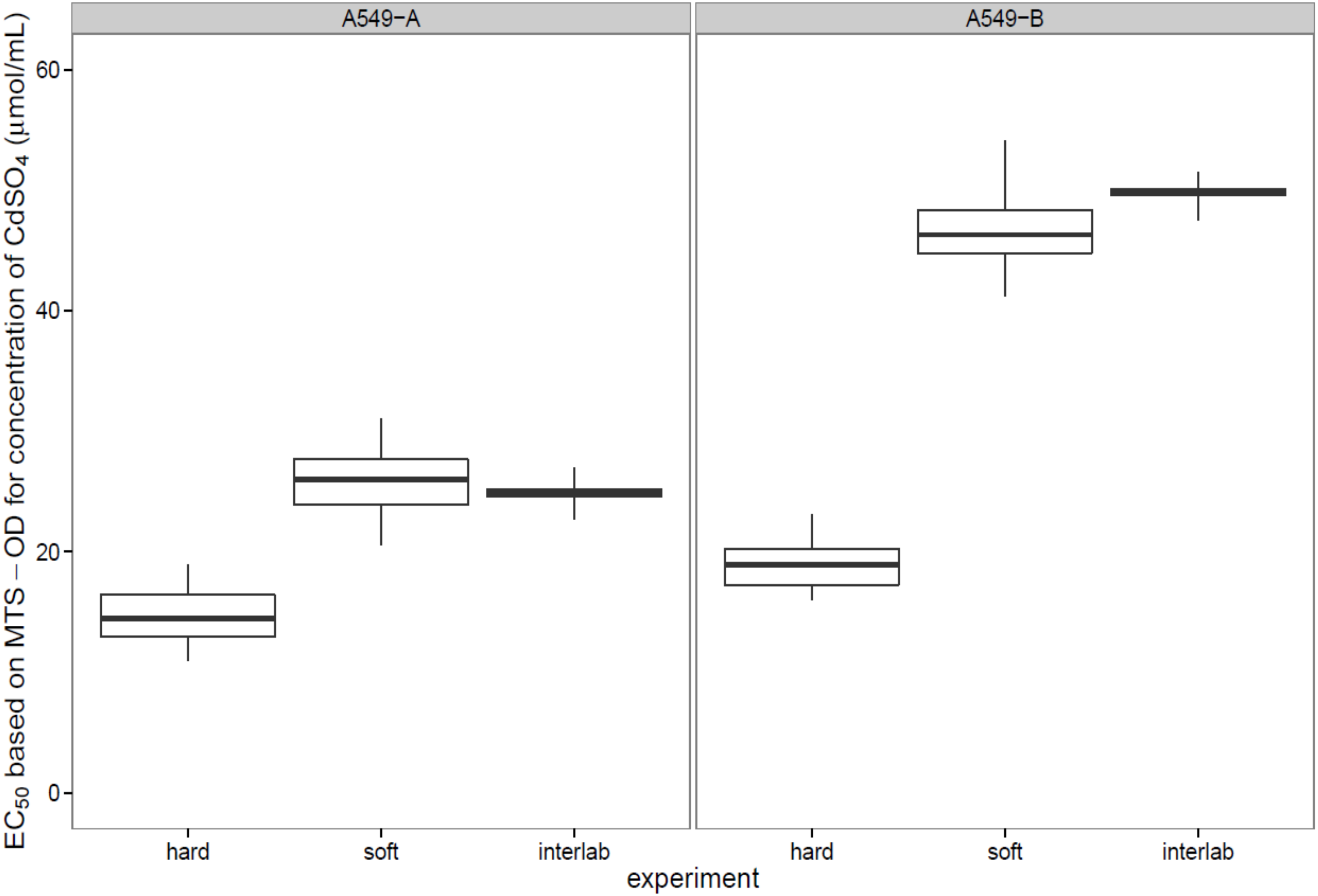


Triplicate reference chemical control. Shows that the assay worked as expected.

Design Element 5: Chemical Control dose response



Design Element 5: Chemical Control dose response



Specification of process controls:

Control	Serum free			Serum		
	target value	range	variability	target value	range	variability
Control 1 (within) B6 – G6	1.8 OD	1.5-2.0 OD	<10%	2.0 OD	1.8-2.3	<7%
Control 2 (between) B3-B6 B8-B10	1.5 OD	1.3-1.8 OD	<12%	2.2 OD	1.8-2.8	<7%
Control 3A Background B7-G7	0.06 OD	0.05-0.09 OD	< 6%	see serum free		
Control 3B [1] Background Chemical Control B2-G2	0.06	0.05-0.09	<6%	see serum free		
Control 3C [2] Background NP B11-G11						
Control 4 [3] Chemical reaction control	49.9	47.5-51.5		77.2	54.3-99.4	

1) If no additional background from the chemical reaction control is observed
2) No values given, because some of the laboratories observed a background signal under serum condition due to NP agglomerates sedimentation
3) Values of the NIST cell line are given. They are fresh out of storage from ATTC and

EVALUATION OF A STANDARD METHOD WITH *C. ELEGANS*

Environmental
Science
Nano

ROYAL SOCIETY OF CHEMISTRY

PAPER

CrossMark
click for updates

Cite this: *Environ. Sci.: Nano*, 2016, 3, 1080

Feasibility of using a standardized *Caenorhabditis elegans* toxicity test to assess nanomaterial toxicity†

S. K. Hanna,*[a] G. A. Cooksey,[a] S. Dong,[ab] B. C. Nelson,[a] L. Mao,[b] J. T. Elliott[a] and E. J. Petersen[a]

Shannon Hanna

Main focus was to evaluate the robustness of an ISO standard method with nanoparticles using a measurement science approach

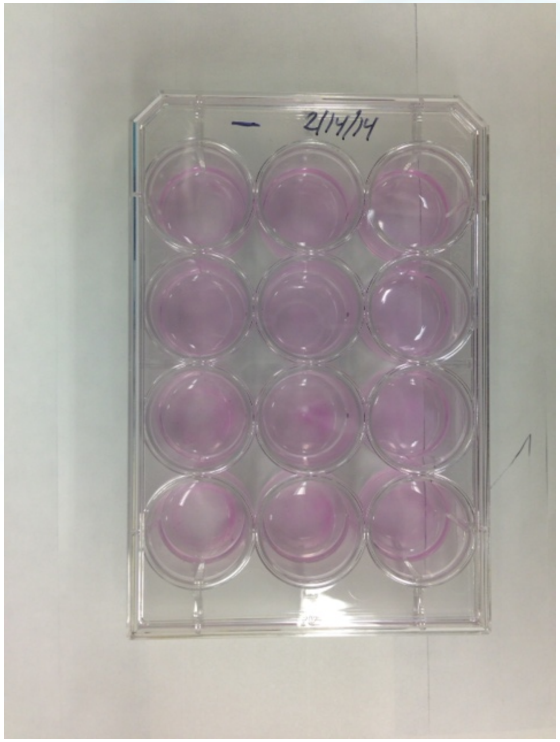
ISO Method 10872

Preparation

1. Add *E. coli* to Luria Broth and incubate at 37°C for 17 h
2. Plate *E. coli* on NGM and incubate overnight
3. Add Dauer larvae to plate and incubate for 72 h

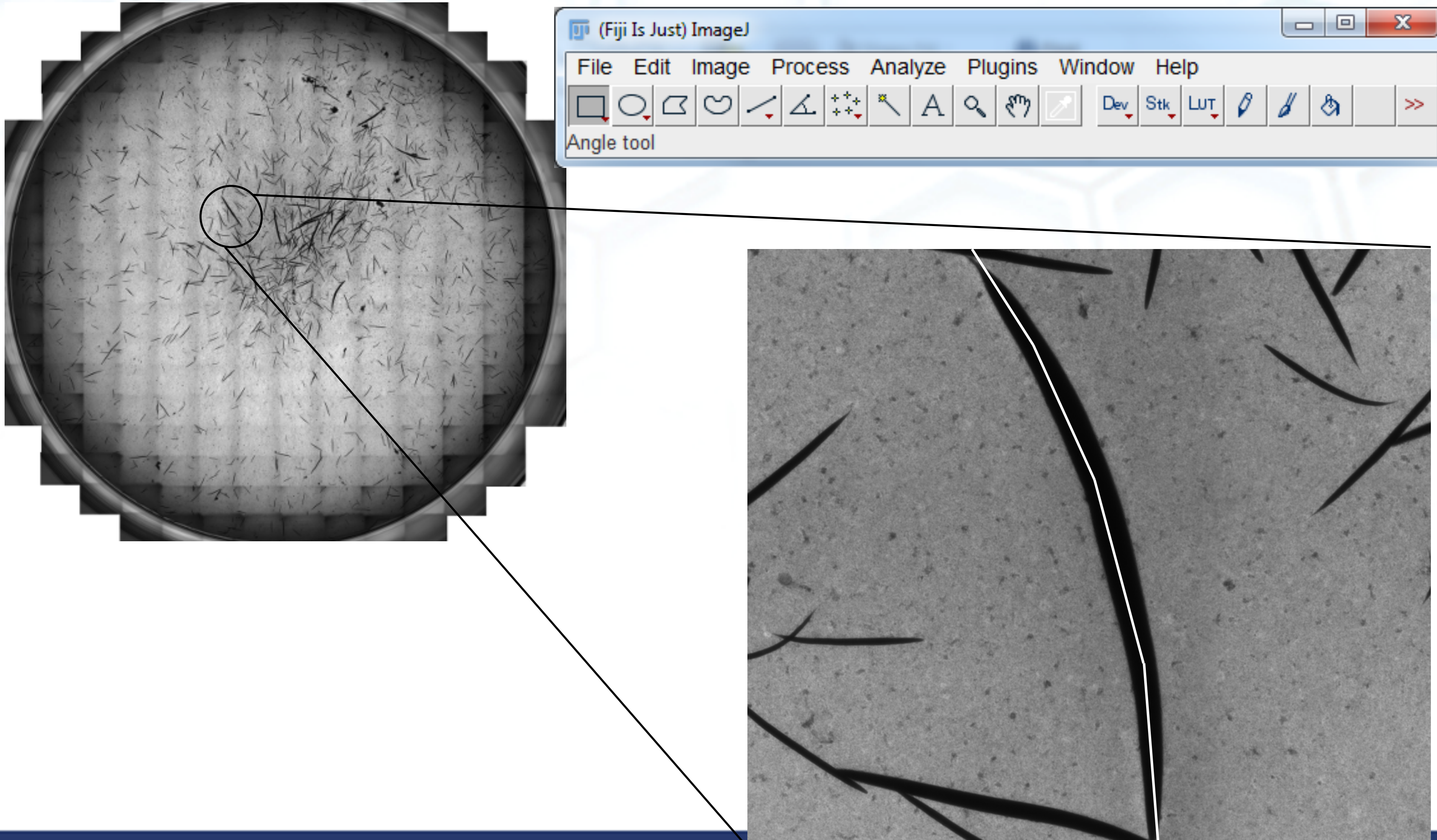
Assay

1. Add chemicals, *E. coli*, and J1 nematodes to 12 well plate, incubate at 20°C for 96 h
2. Add 200 ul Rose Bengal, heat plate at 80°C for 10 min
3. Allow plate to cool, add 1ml of mineral oil, image plate, and analyze images

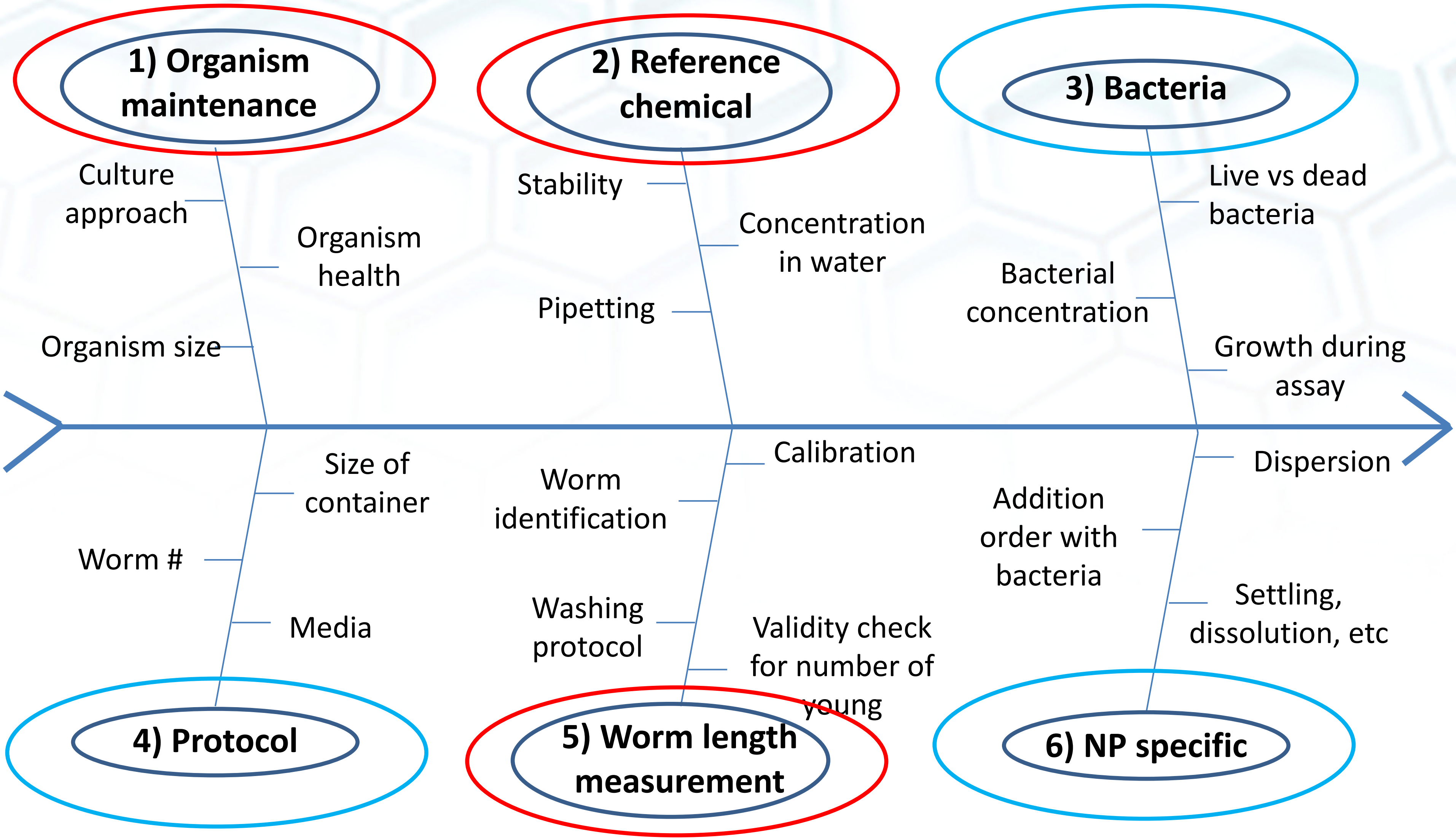


- Uses positive control benzylcetyldimethylammonium chloride (BAC C16 – EC_{50} = 15.1 mg l^{-1})
- Only test specification is growth inhibition of 20-80% at 15 mg l^{-1}

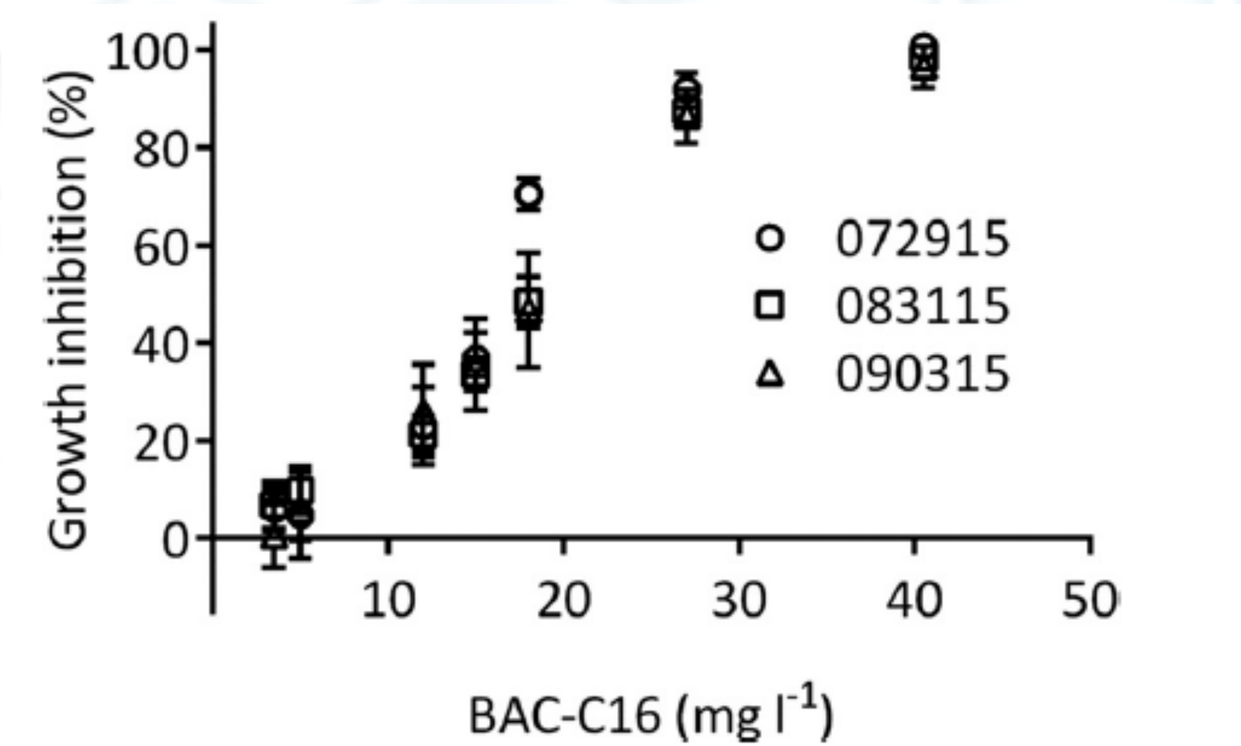
QUANTITATIVE MICROSCOPY



Cause & Effect Analysis of *C. elegans* Assay

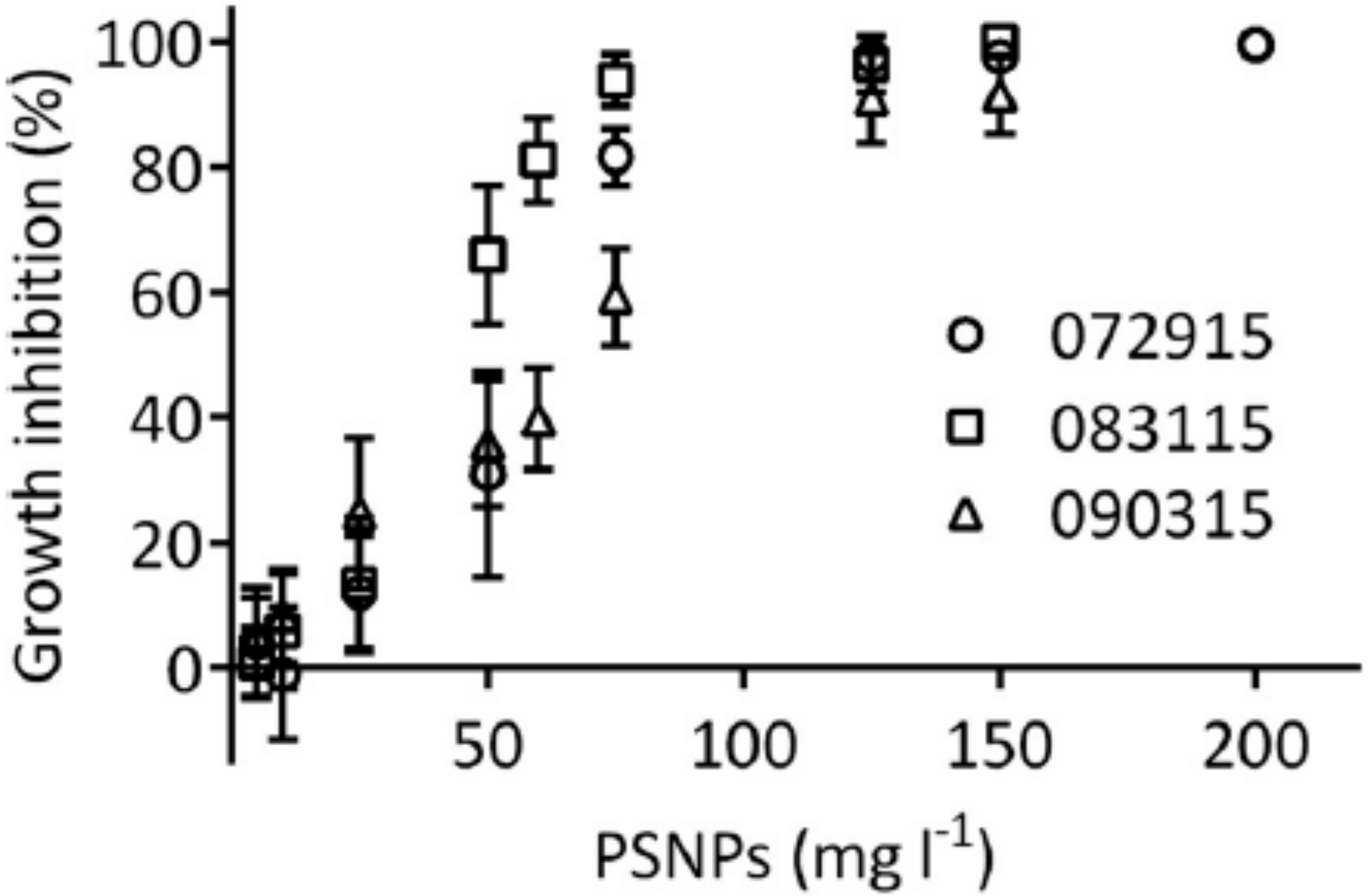


Reproducibility with BAC-C16



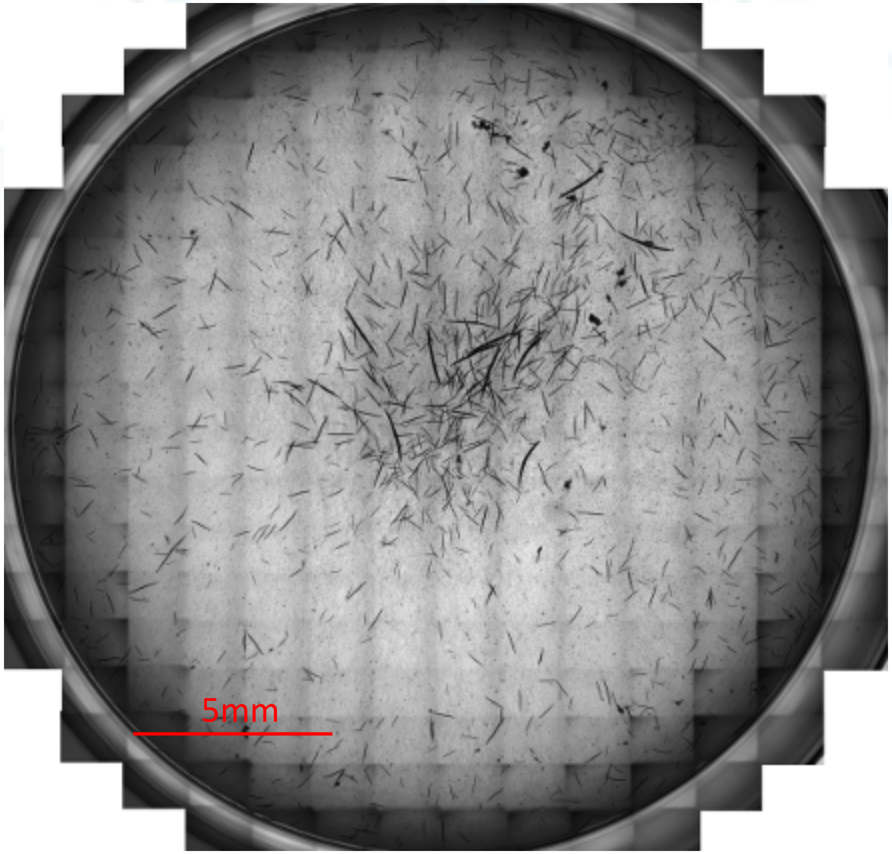
EC$_{50}$ for growth = 18.7 ± 2.6 mg l^{-1}

Reproducibility with PSNPs

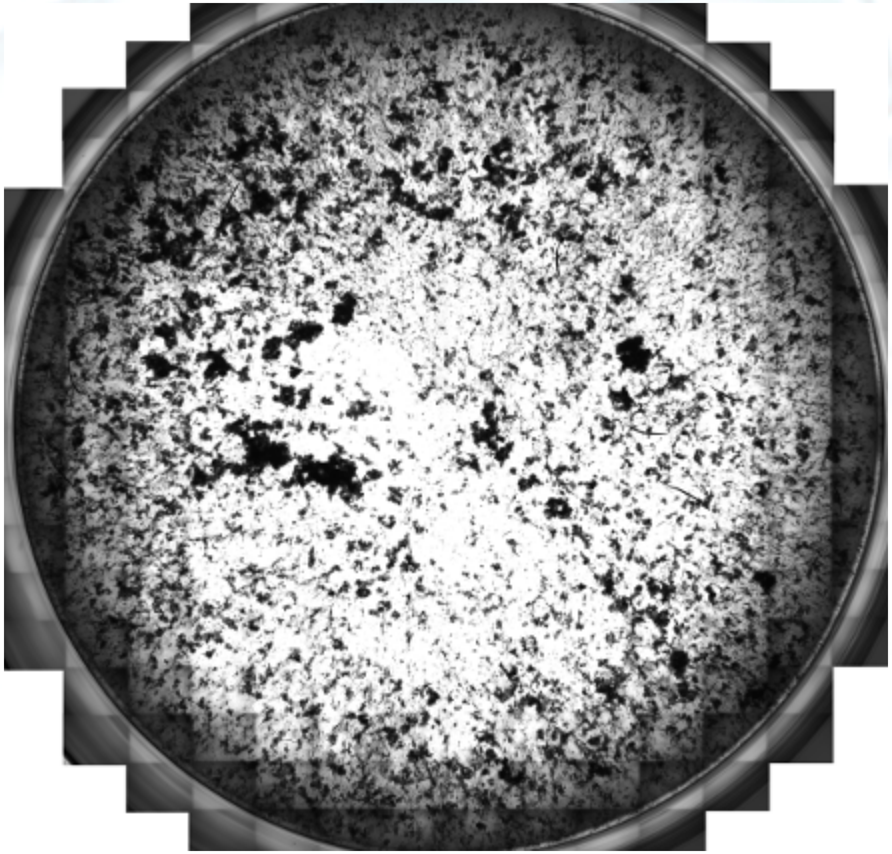


EC_{50} for growth = 71.7 ± 37.2 mg l^{-1}

Images of plates



Control



100 mg L^{-1} PSNPs

Environmental Science & Technology

Article

Cite This: Environ. Sci. Technol. 2018, 52, 5968−5978

pubs.acs.org/est

Agglomeration of *Escherichia coli* with Positively Charged Nanoparticles Can Lead to Artifacts in a Standard *Caenorhabditis elegans* Toxicity Assay

Shannon K. Hanna,[*,#] Antonio R. Montoro Bustos, Alexander W. Peterson, Vytas Reipa, Leona D. Scanlan,[†] Sanem Hosbas Coskun, Tae Joon Cho, Monique E. Johnson, Vincent A. Hackley, Bryant C. Nelson, Michael R. Winchester, John T. Elliott, and Elijah J. Petersen

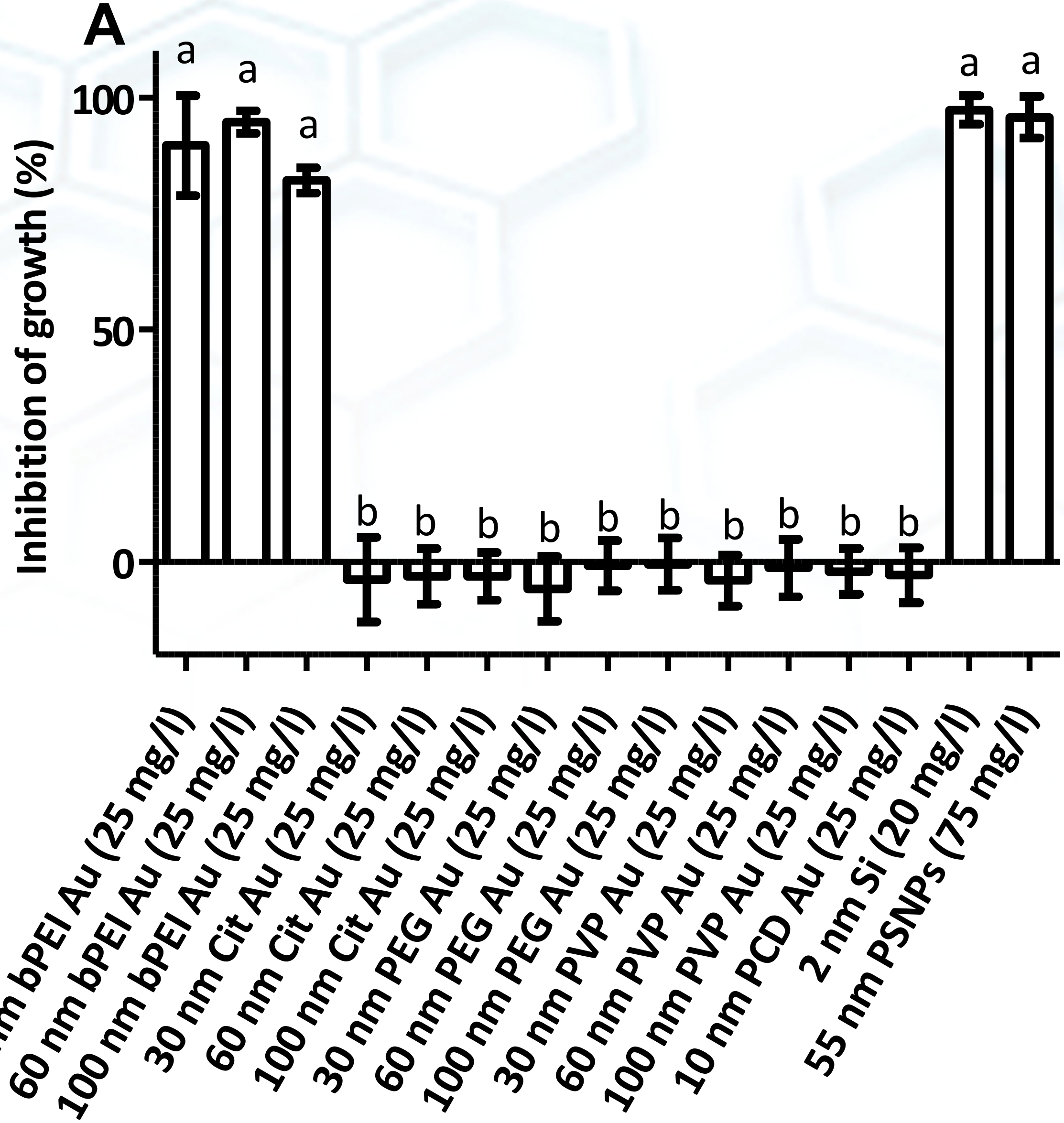
Is this assay robust when tested with a broader range of nanoparticles?

PSNPs – amine coated, 55nm

Si NPs – amine coated, 2nm

Au NPs – various coatings (PVP, PEG, Citrate, bPEI, dendron) and sizes (10-100nm)

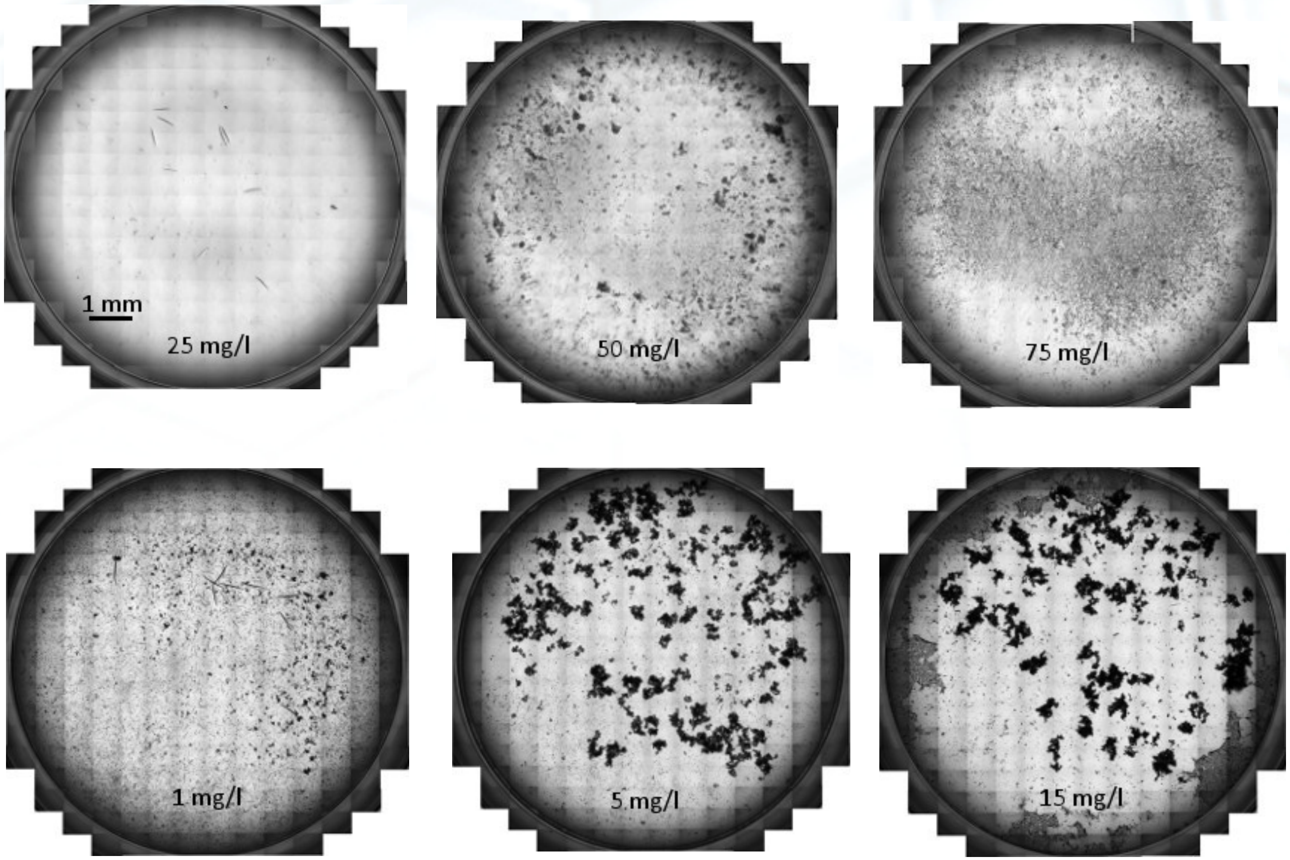
NP toxicity to *C. elegans* ISO 10872



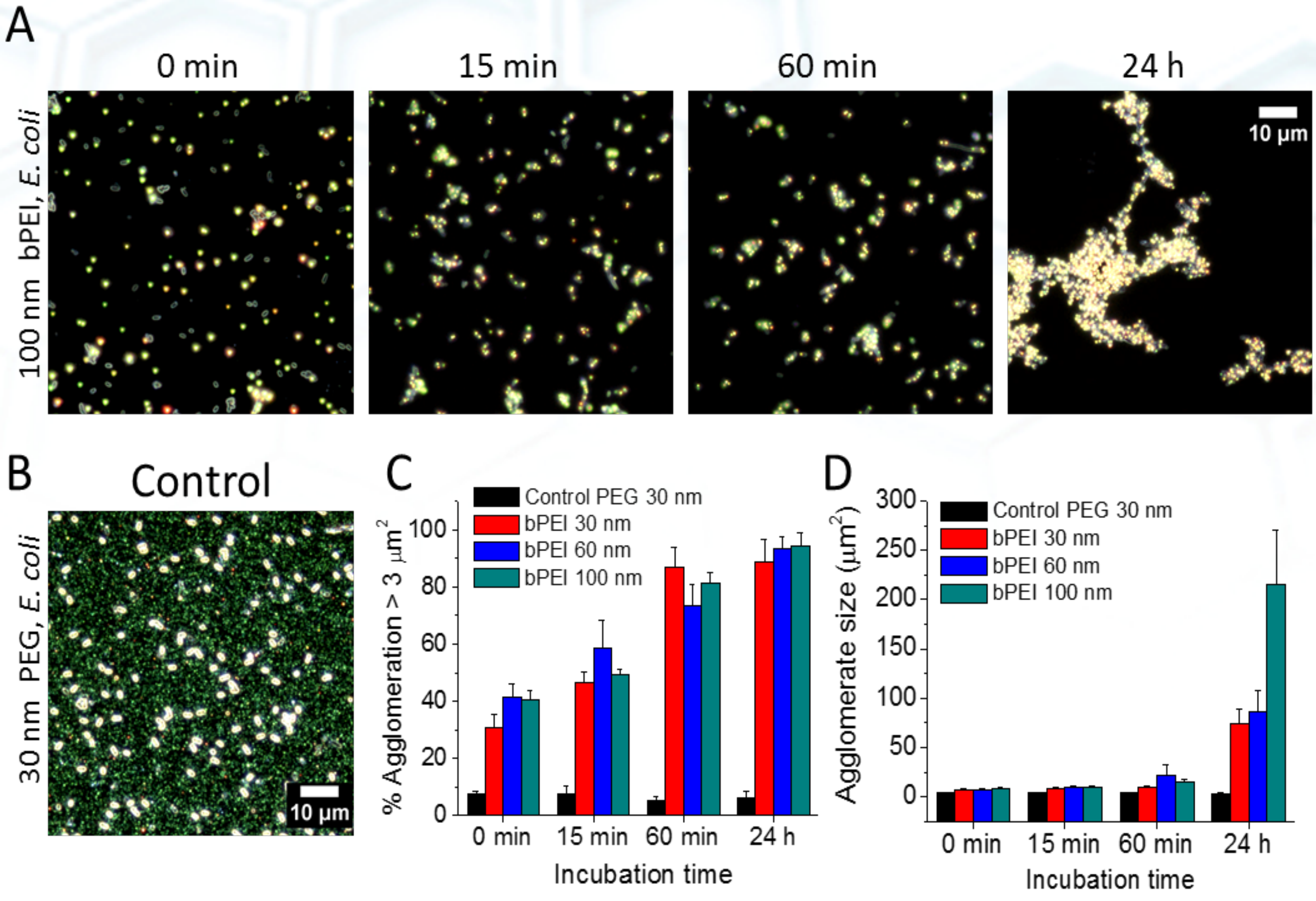
Light microscopy analysis

PSNPs

bPEI AuNPs

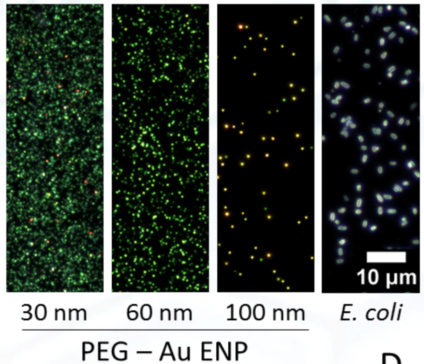


Heteroagglomeration of Positively Charged Nanoparticles with *E. coli* using enhanced darkfield microscopy

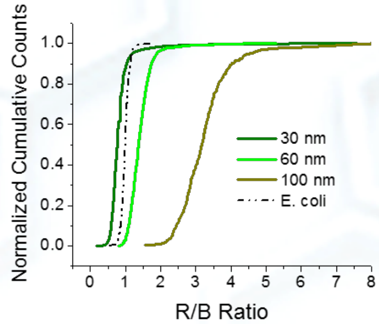


Heteroagglomeration of Positively Charged Nanoparticles with *E. coli* using enhanced darkfield microscopy

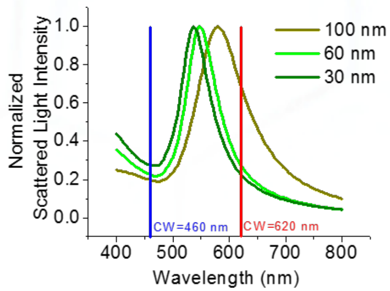
A



B



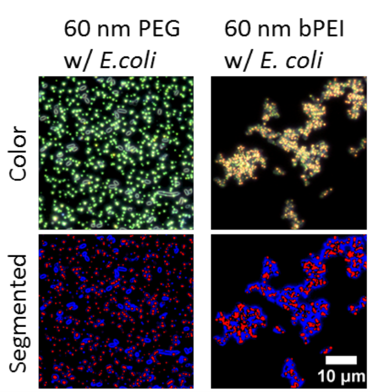
C



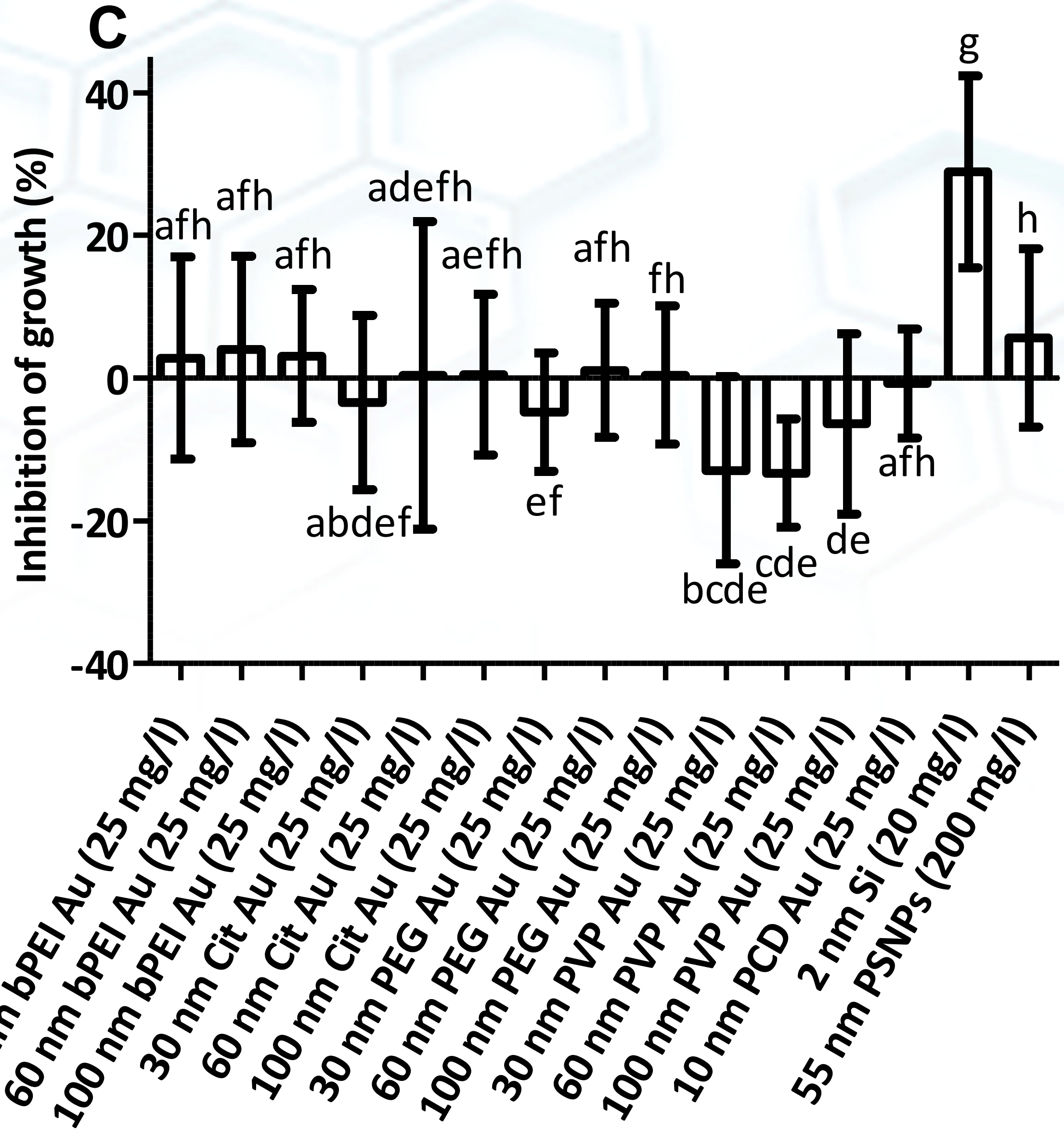
D

R/B ratio					
	Mie theory	PEG	PVP	CIT	bPEI
30 nm	0.83	0.82 ± 0.29	0.83 ± 0.17	2.63 ± 0.66	1.24 ± 0.40
60 nm	1.22	1.44 ± 0.32	1.39 ± 0.24	2.76 ± 0.67	1.83 ± 0.77
100 nm	3.53	3.55 ± 0.79	3.61 ± 0.84	3.09 ± 0.99	3.30 ± 0.87

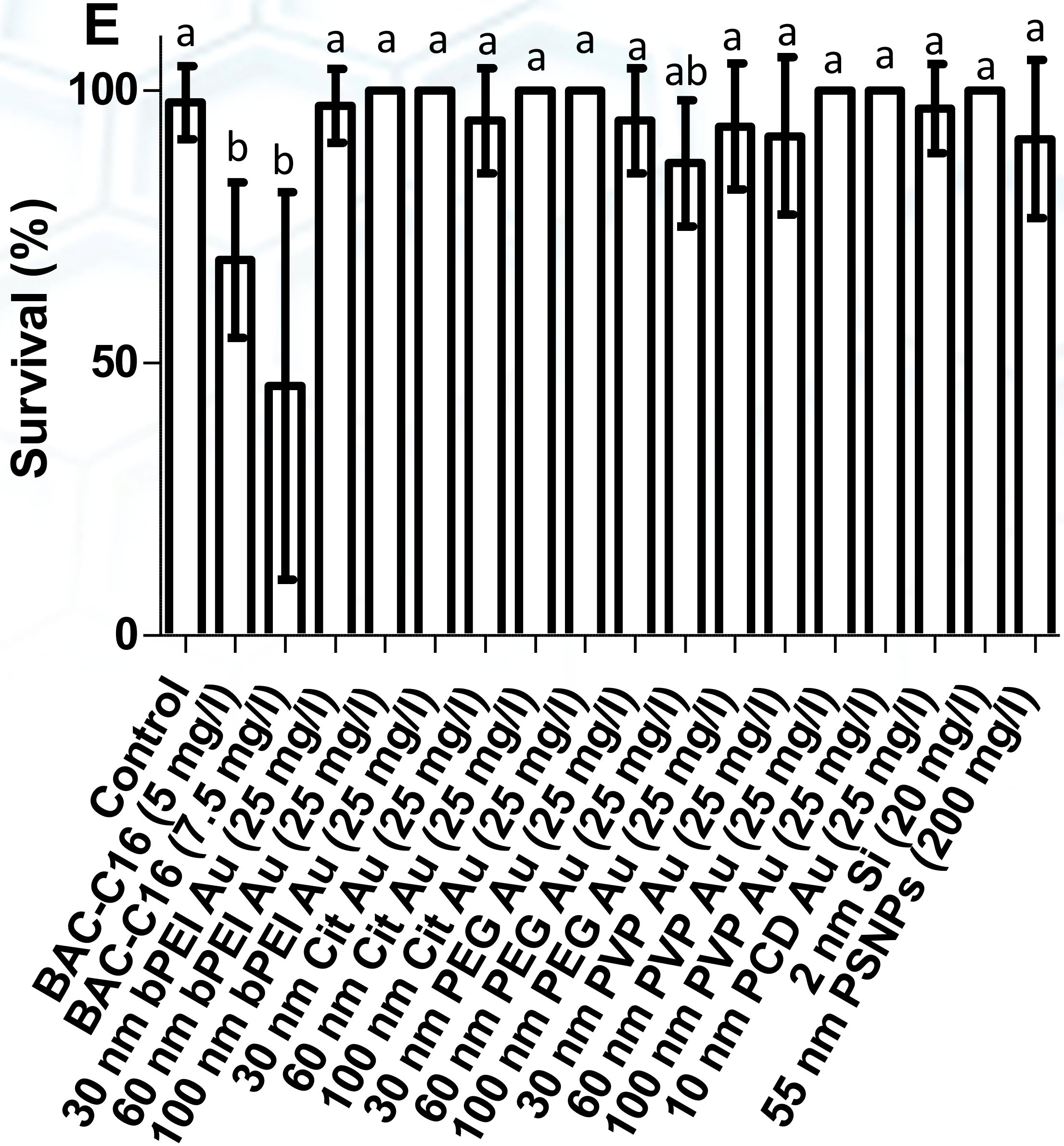
E



NP toxicity to *C. elegans* using an axenic medium



NP toxicity to *C. elegans* using a water-only Mortality Assay



Conclusions

Many potential artifacts in nanotoxicology are now known and control experiments are defined.

Measurement science tools have been used to quantify sources of uncertainty in a *C. elegans* assay and the MTS cytotoxicity assay (now published as an ISO standard).

Comparability of data among experiments and between laboratories can be evaluated through process control measurements. These process control measurements need to cover potential artifacts and biases that can occur during the assay, and also interactions among variables (e.g., change in cell number changing the EC_{50} value).

NIST Collaborators

Tae Joon Cho
Greg Cooksey
John Elliott
Vincent Hackley
Shannon Hanna
Sanem Hosbas Coskun
Monique Johnson
Teresa Kirschling
Robert MacCuspie
Antonio Montoro Bustos
Karen Murphy
Bryant Nelson
Alex Peterson
Vytas Reipa
Marc Salit
Fatima Sequeira
Leona Scanlan
Blaza Toman
Michael Winchester

External collaborators

Heriot-Watt University
Theodore Henry

University of Massachusetts
Jian Zhao
Baoshan Xing

Nanotechnology Characterization Laboratory
Marina Dobrovolskaia

Connecticut Agricultural Experiment Station
Jason White

National Nanotechnology Center
Rawiwan Maniratanachote
Wongsakorn Suchaoin

Ecole Polytechnique Federale de Lausanne
Nadia von Moos

EMPA
Matthias Roesslein
Anna Neuer
Peter Wick
Cordula Hirsch
Harald Krug

Nanjing University
Shipeng Dong
Liang Mao

Korea Research Institute of Standards and Science
Nam Woong Song
Soo Jin Kim
Jieun Lee

JRC
Agnieszka Kinsner-Ovaskainen
Francois Rossi