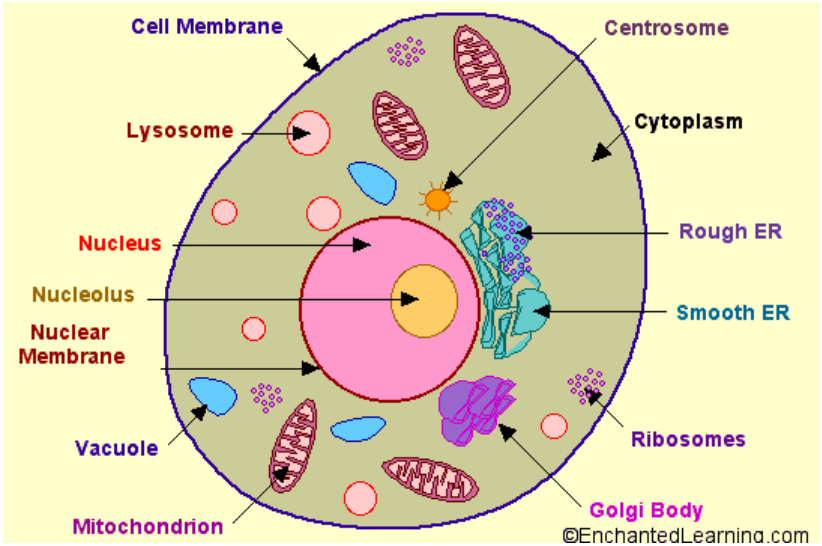
Issues in validating cell-imaging based assays

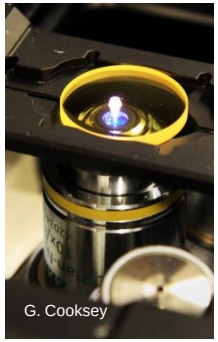


http://www.enchantedlearning.com/subjects/animals/cell/

J. Elliott, A. Plant, M. Halter, G. Cooksey, K. Bhadriraju, A. Tona
Cell System Sciences Group
NIST Gaithersburg
AIMBE/NIH Summit, March 19, 2012

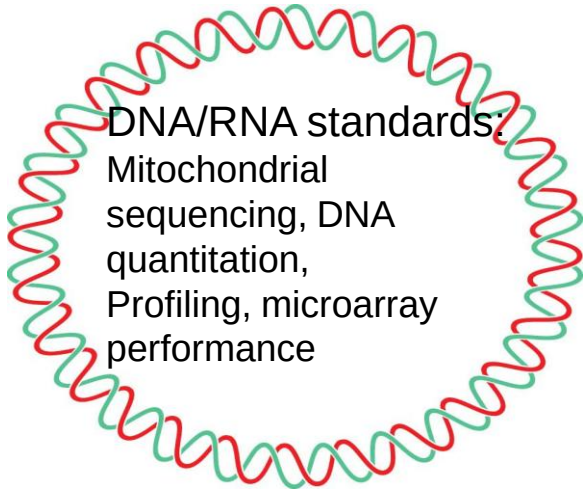
What is NIST….

-Department of Commerce
-Mission to improve American life via good measurements
-Standards, new instrumentation and new measurement strategies



Optical standards:
ND Filter, fluorescence, spectral

Chemical standards:
Vitamin D, hormone in serum, albumin



DNA/RNA standards:
Mitochondrial sequencing, DNA quantitation, Profiling, microarray performance

ASTM
CLINICAL AND LABORATORY STANDARDS INSTITUTE
ISO

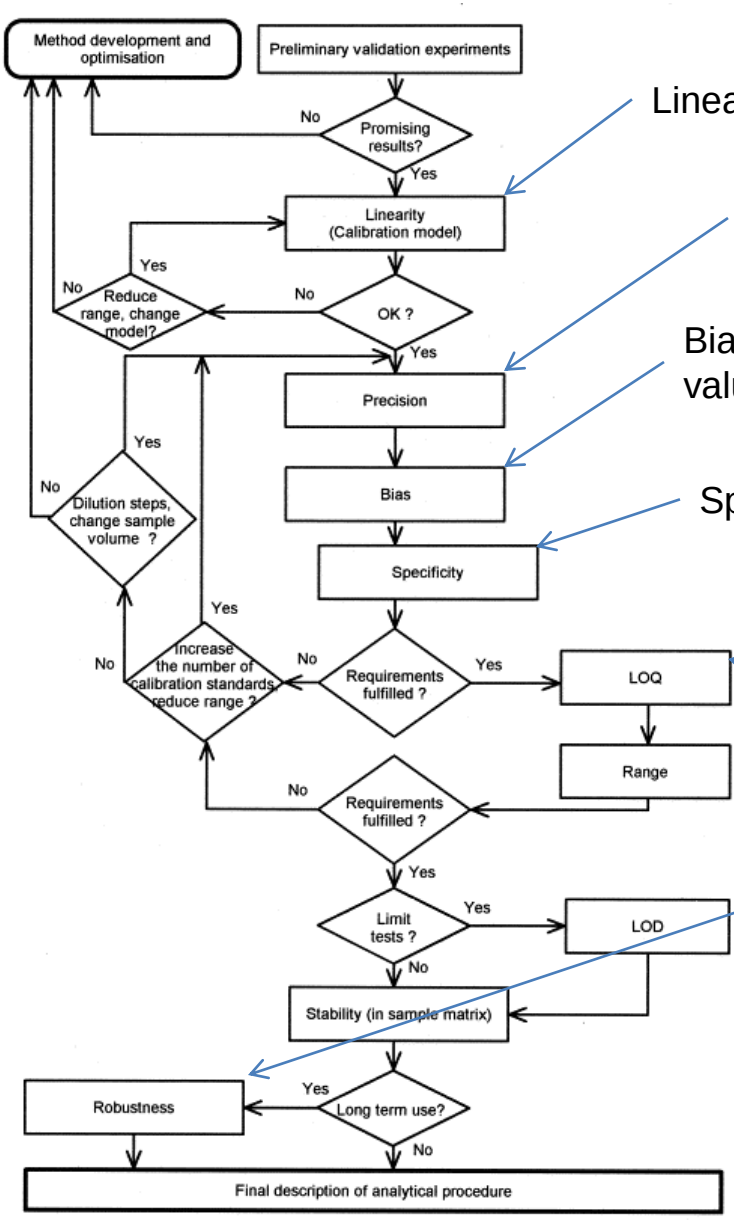
Documentary standards

Validation of a measurement

Is the measurement right? What's the evidence?

- Measurement characteristics
  - **Accuracy**- How close to the real value?
  - **Precision**- how much random error?
  - **Response function-** linearity/calibration
  - **Reproducibility**-do you get the same measurement next week?
  - **Robustness**-sensitivity to variations in assay parameters
- Instrument performance characteristics
  - **Dynamic range-** lowest limit of detection, highest limit of detection
  - **Contrast transfer function-** signal to noise, resolution
- Standards, reference materials and protocols
  - Reference materials- flatfield standard, fluorescence standard
  - Quantitative imaging protocol (i.e. flatfield, dark counts, etc)
  - Positive and negative controls, calibration curve

Example: Bioanalytical Validation flowchart (analyte concentration)



Linearity/calibration model- identify and test a range of concentrations

Precision- Repeat measurements 3x with independent samples

Bias/Accuracy- compare results against standard value (orthogonal method)

Specificity- does matrix influence the measurement

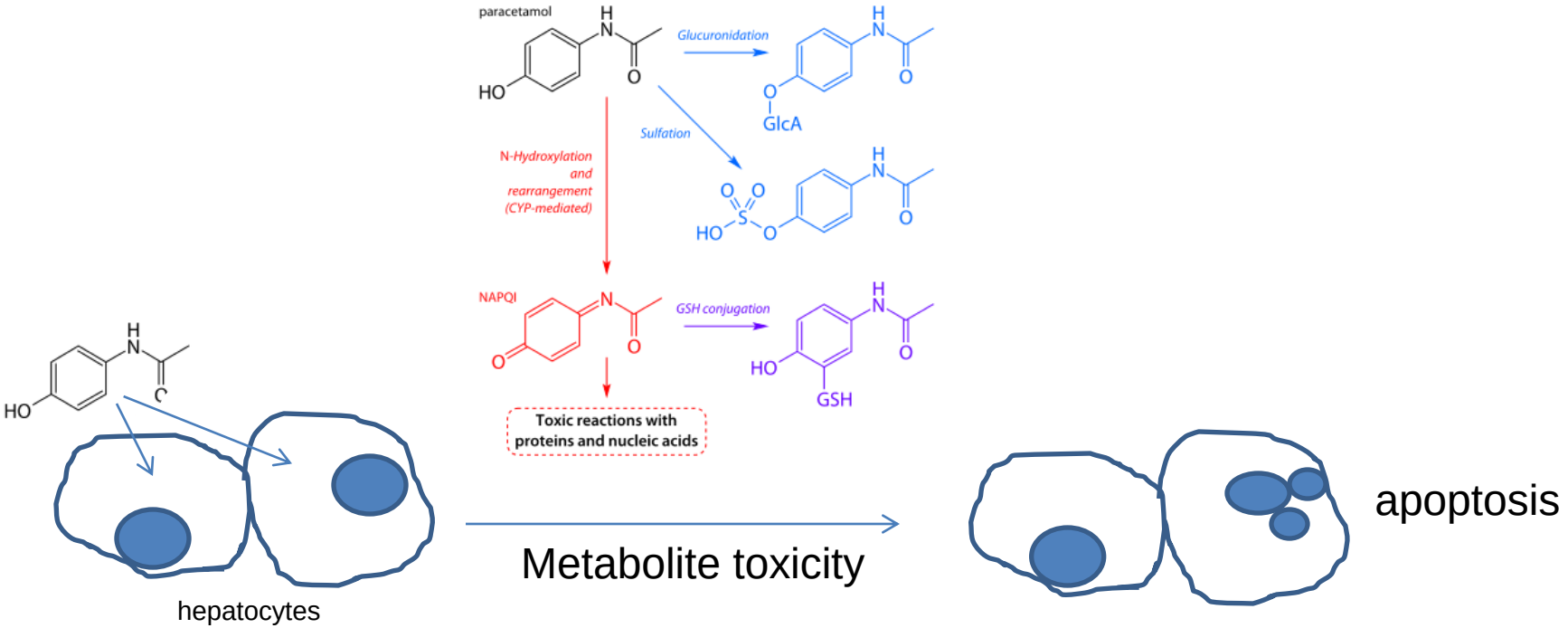
Limit of quantitation (LOQ)- predict and test

Robustness- use experimental design to identify other sources of variability

•Documentation of these parameters provides evidence of a validated assay measurement

C. Hartmann et al. J. Pharmaceut. Biomed. Anal. 1998, 193-218

Validation of biological function

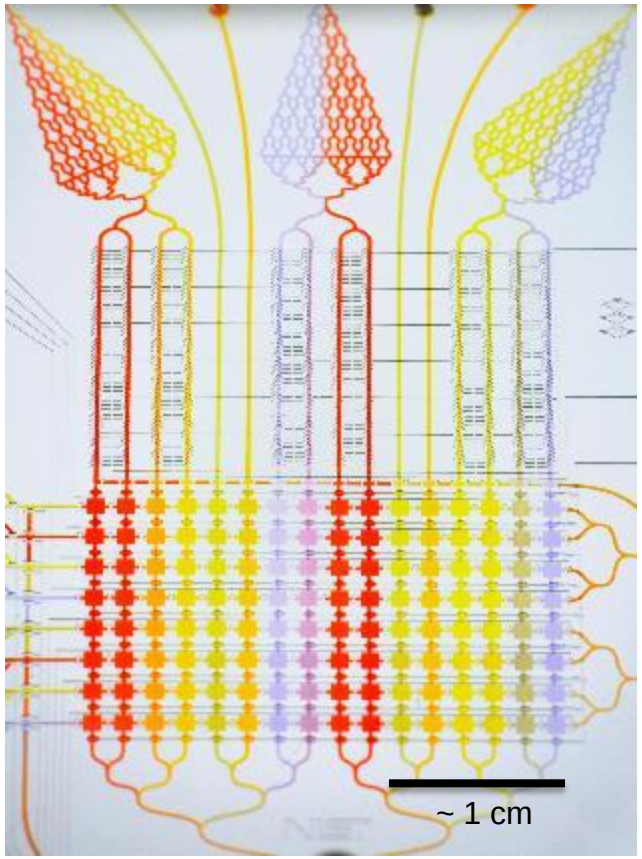


- Is the biological mechanism of action true?
  - Control compounds with known effects (inhibitors, activators)
  - Additional high quality assays and experiments likely required (live cell imaging, knockouts)
  - The aggregate of data validates a biological function.

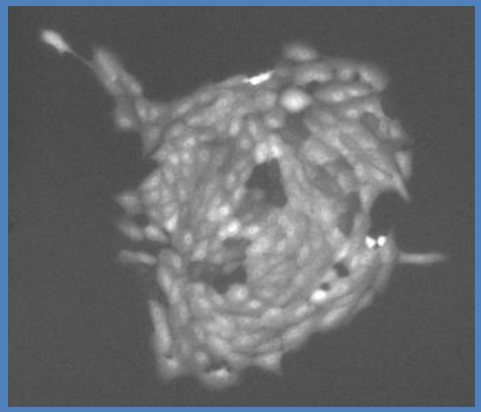
Microfluidic cellular assays

- Advantage: Complex plumbing possible, highly parallel, less reagent
- Disadvantage: not the same as “bulk” TCPS dish gold standard- high surface to volume ratio (leaching), gas permeability, cell seeding

128 chamber device w/ gradient mixer (G. Cooksey)



Ribosome Inhibitor Assay



CMV-dsEGFP-Vero cells
$t_{1/2}$~2h

Validation Issues

Results comparable to TCPS?

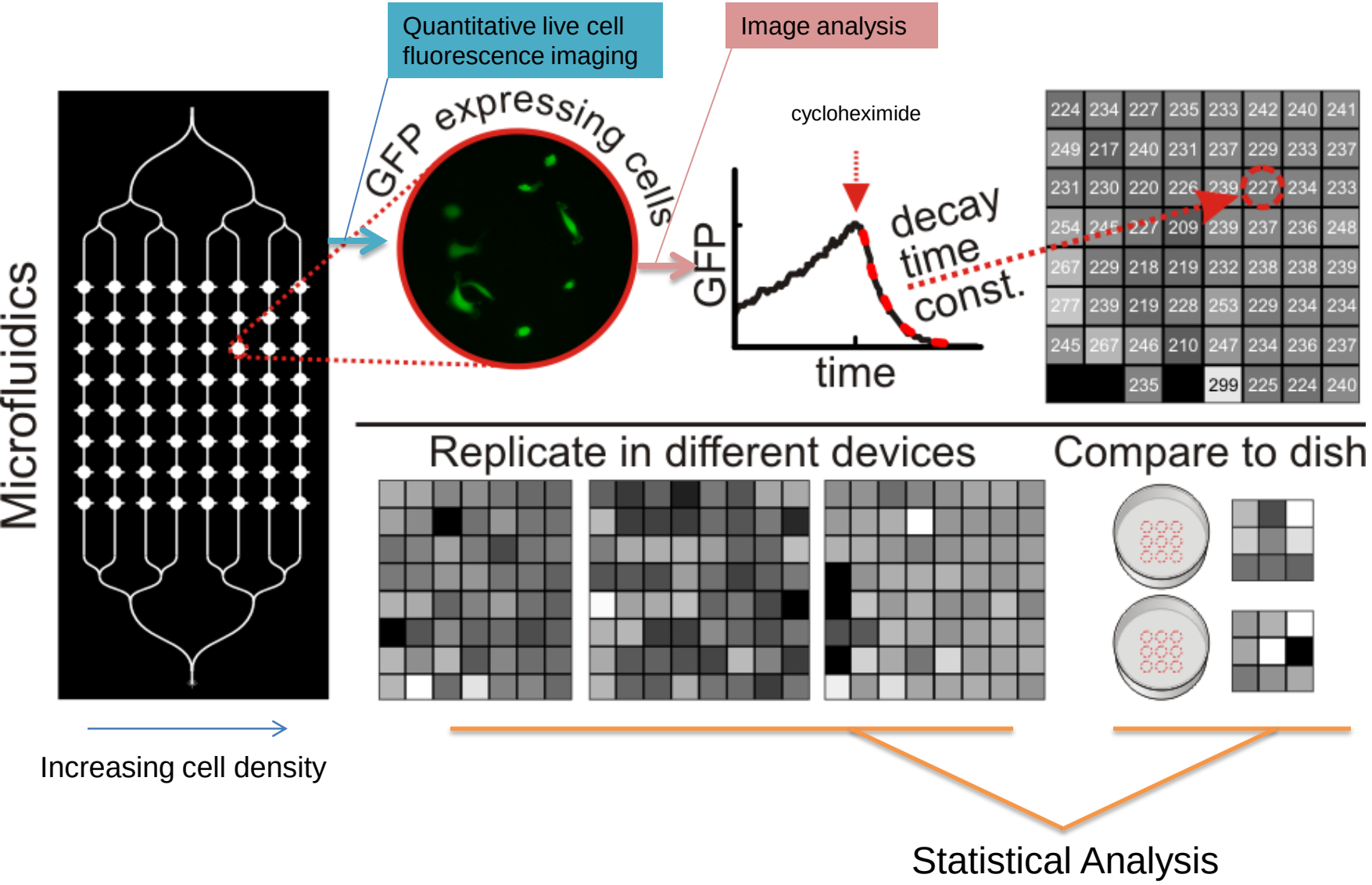
Cell density effects?

Paracrine signaling effects?

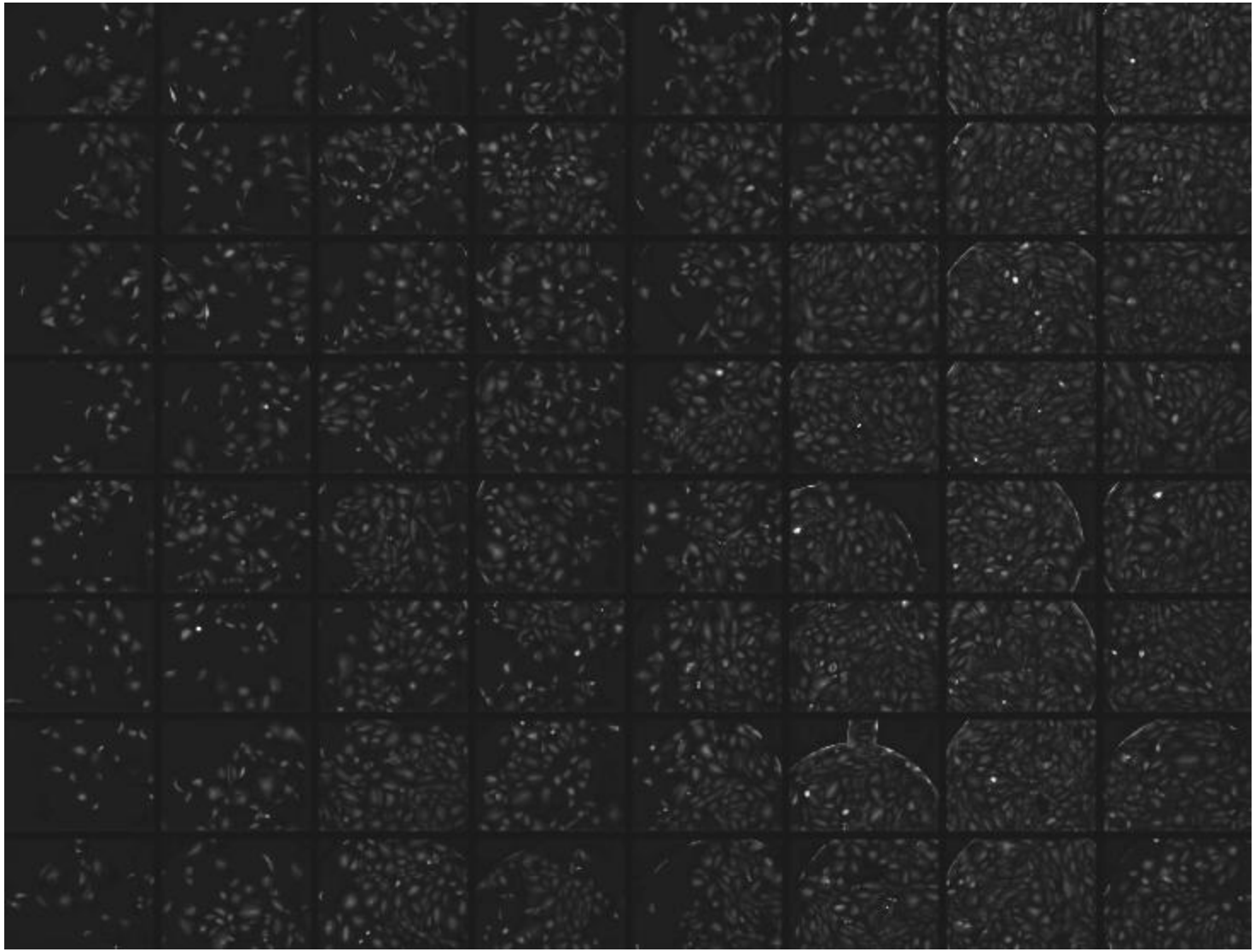
Flow rate, tubing fabrication, imaging platform effects?

Halter et al. Assay Drug Dev Technol. 2009 Aug;7(4):356-65.

Experimental Design for Device Performance Evaluation



Cooksey et al Anal Chem 2011

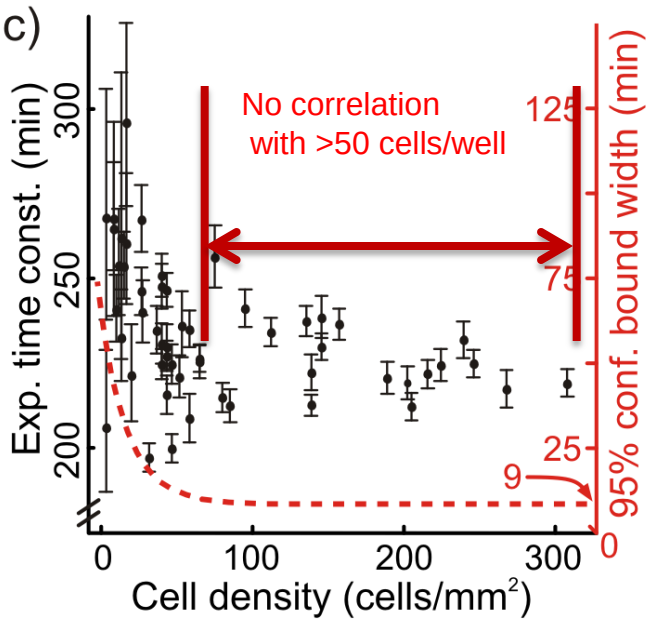


dsEGFP-Vero cells and cycloheximide- originally developed for ricin potency

GFP Decay Assay Performance Summary

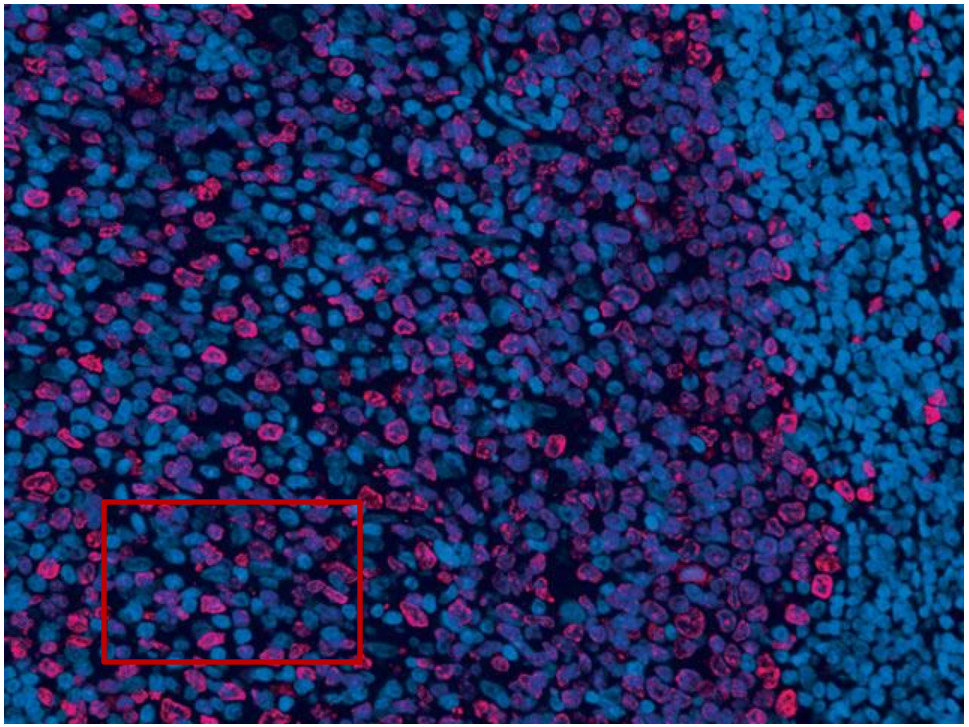
Substrate	Tubing	Lamp	Flow (µl/min)	Mean T± stdev (min)	*n* (# regions)	Signal-to-Noise Ratio
Microfluidic Experiment A	Teflon	LED	0.1-1.5	236 ± 27	54	64 ± 2
Microfluidic Experiment B	Teflon	Hg arc	0.5-3.5	224 ± 31	64	47 ± 3
Microfluidic Experiment C	Tygon®	Hg arc	0.5-2	236 ± 15	61	48 ± 3
Microfluidic* Experiment D	Tygon®	Hg arc	0.5-2	254 ± 19	61	138 ± 50
TCPS dish						
A	N/A	LED	Bulk	244 ± 66	8	14 ± 3
B		Hg arc		239 ± 48	9	7 ± 1
C		Hg arc		215 ± 27	9	9 ± 1
D		Hg arc		250 ± 13	9	9 ± 2
PDMS-coated dish						
A	N/A	LED	Bulk	254 ± 41	9	11 ± 1
B		Hg arc		246 ± 13	9	11 ± 2

N/A = not applicable
Bulk = 3ml stagnant media
*mean ± std (5 to 15h)

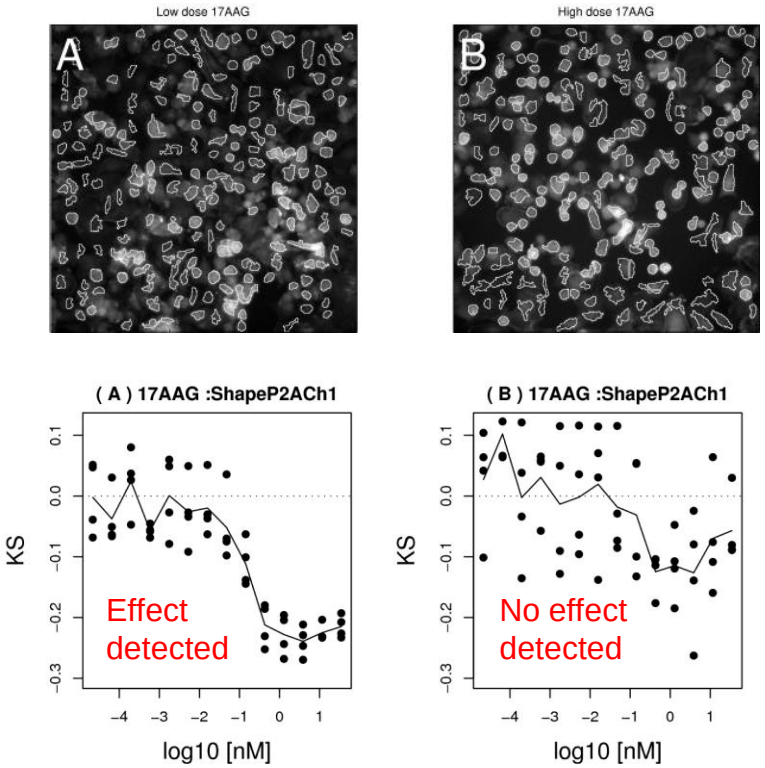


Questions:	Evidence
Comparable to TCPS?	Yes. No statistical difference.
Cell density effects?	No after 50 cells/well
Well position effects?	No. No statistical difference.
Robust?	Robust to fabrication, tubing, lamp, flow rate, replication
Other information:	5-fold higher signal to noise

Evaluating the Accuracy of Image Analysis Tools



Tonsil tissue w/DAPI and Ki-67 antibody (Biocare Medical)



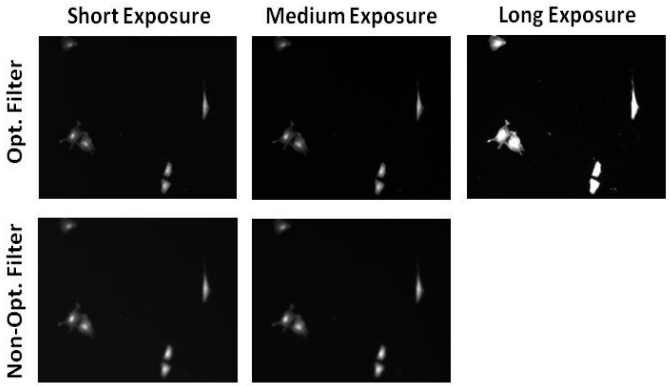
Hill et al BioMed Central Bioinformatics 2007

- What do you want to quantify (metric)?
- How many images are required?
- What image analysis do you use?
- What validation evidence do you have?

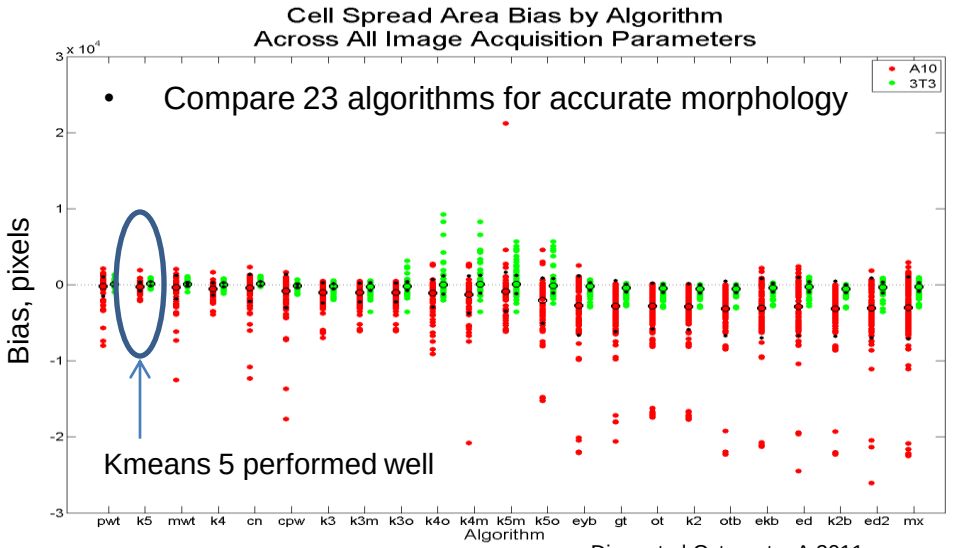
- Inaccurate image analysis can lead to erroneous conclusion!!

Evaluation the Accuracy of Cell Segmentation

Fluorescent images

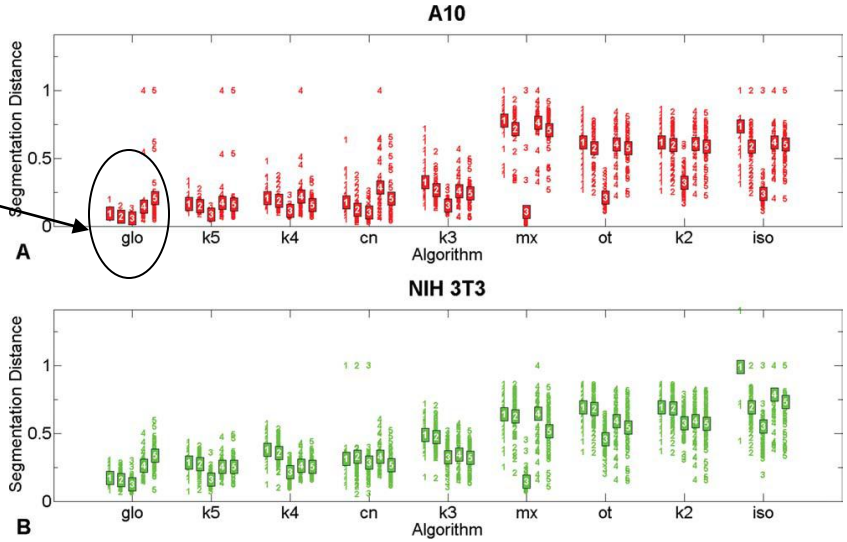


Vary experimental acquisition conditions



Dima et al Cytometry A 2011

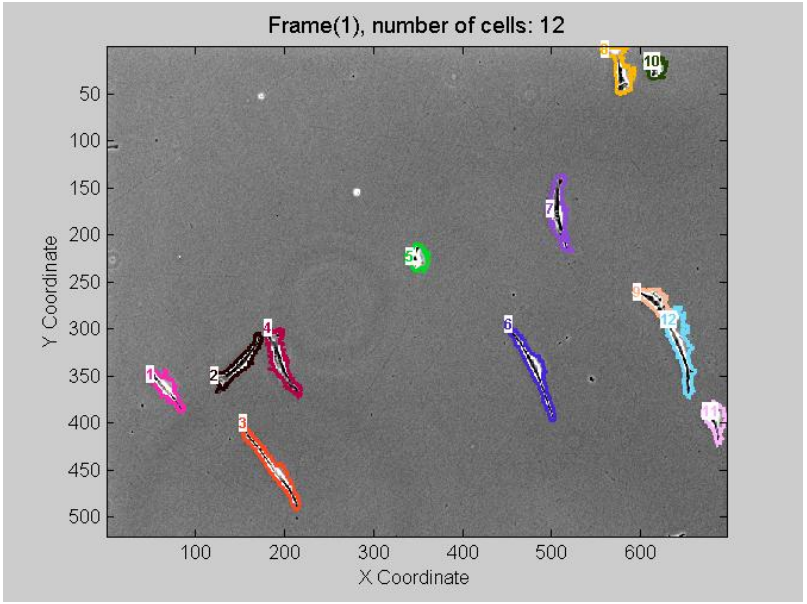
Robust over imaging conditions



- Requires manual (expert-trained) segmentation data to serve as reference data
- Image series, benchmarks, manual segmentation databased at www.sbd.gov

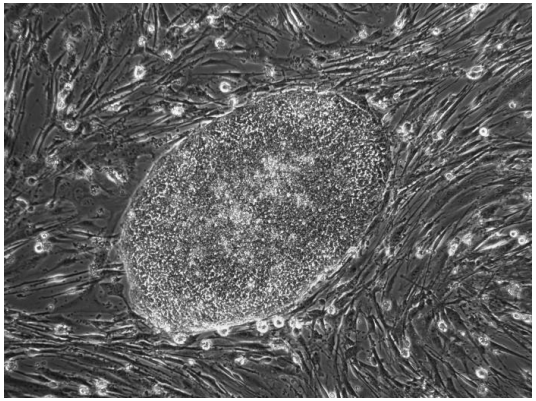
Non-destructive Phase Imaging

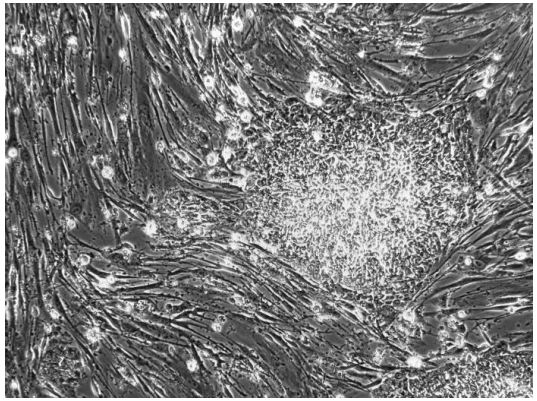
Phase imaging, NIH 3T3



- Phase can be used for live cell imaging.
- Segmentation algorithm custom
- Need to determine acceptance criteria

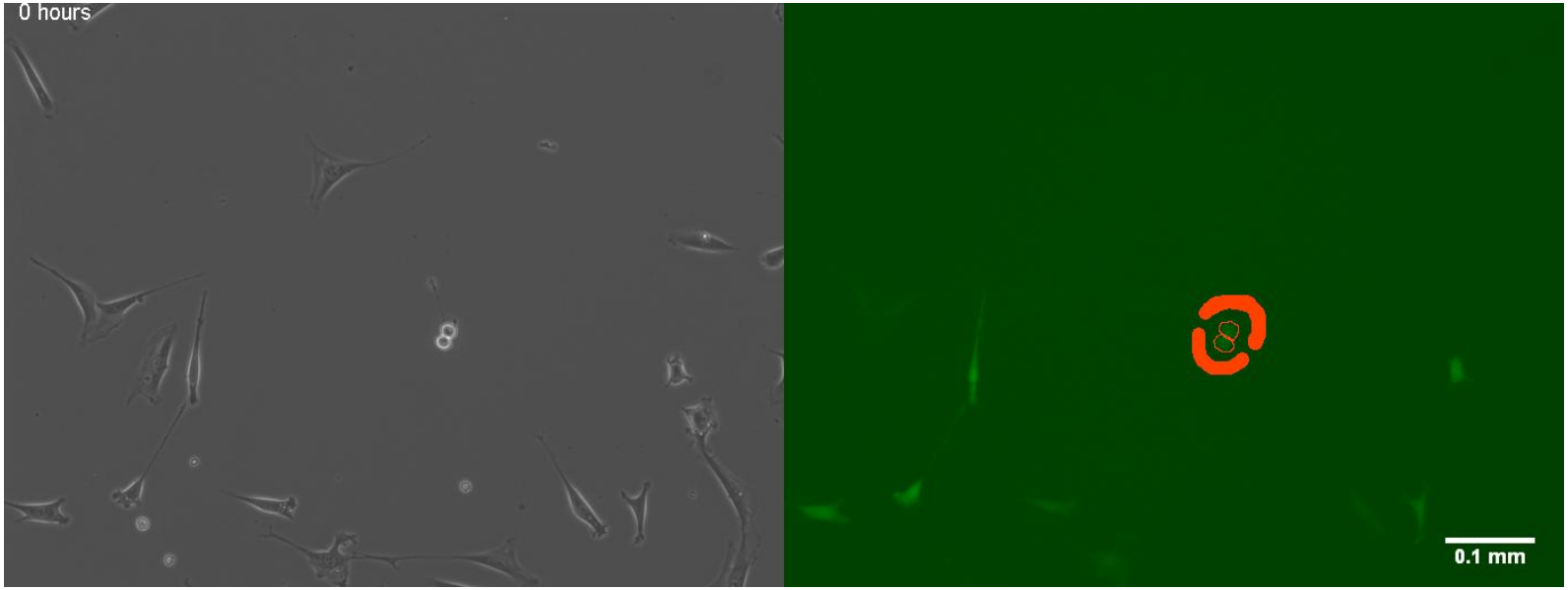
Pluripotent stem cells





Differentiation in stem cells

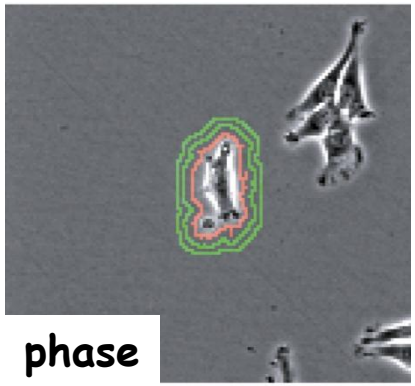
Quantifying GFP reporter activity in live cells

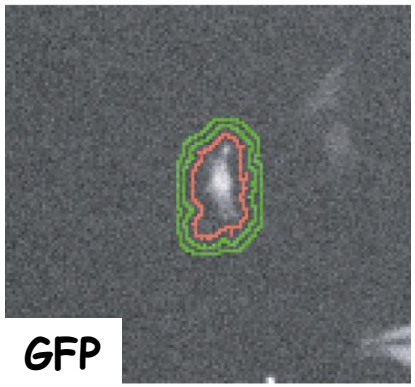


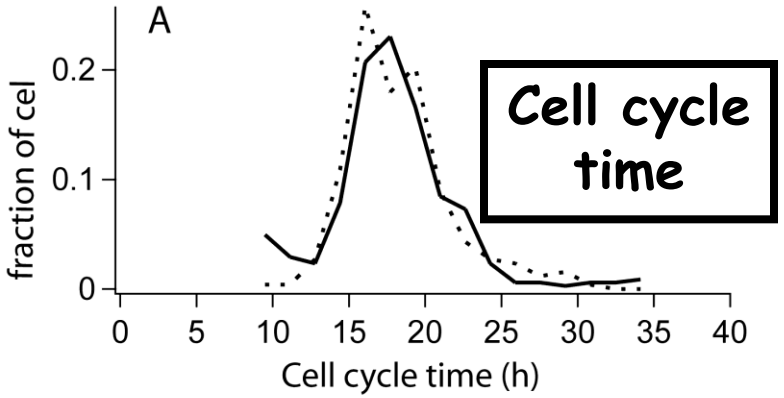
CTRL: Flatfield corrected with 50% fluorescein solution
CTRL: <3% photobleaching of GFP over imaging time
CTRL: Stable lamp intensity (LED)
97% complete-cell-cycle accuracy (193 tracked cells out of 199)
99.92% frame-to-frame accuracy (6 missed tracks out of 7957)

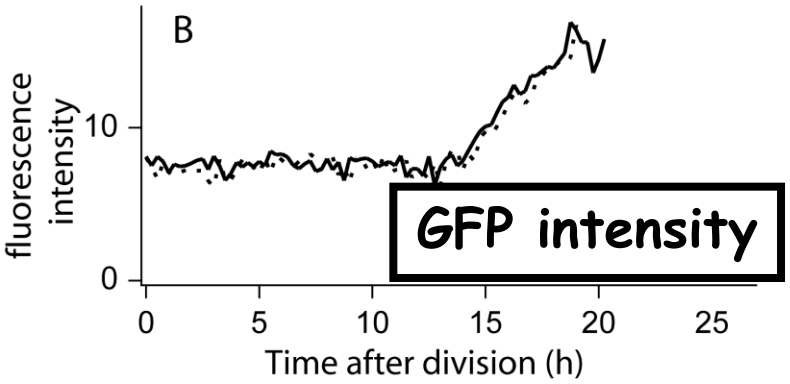
Validation of automated algorithms

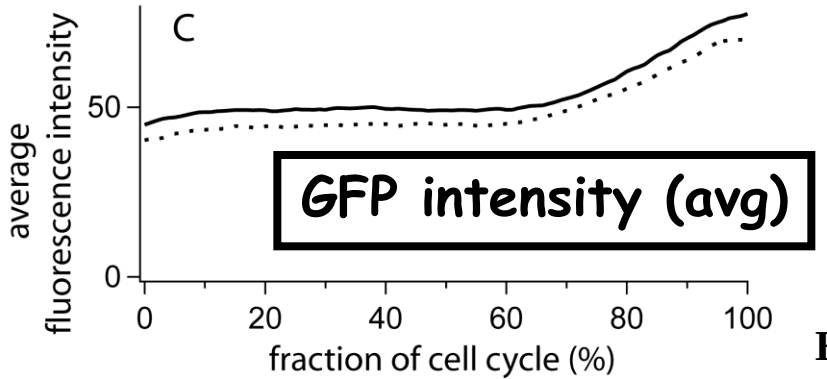
Automated analysis
n=344



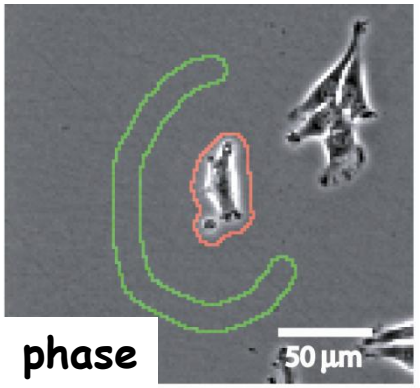


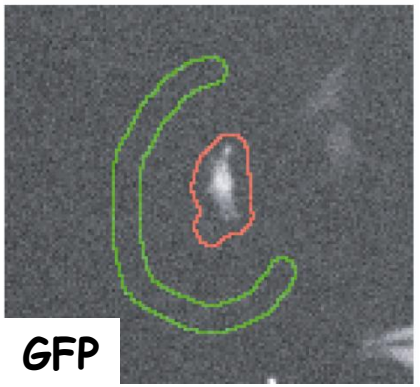






Manual analysis
n=257





Adjusted Rand Index
0.956
(7,186 masks)

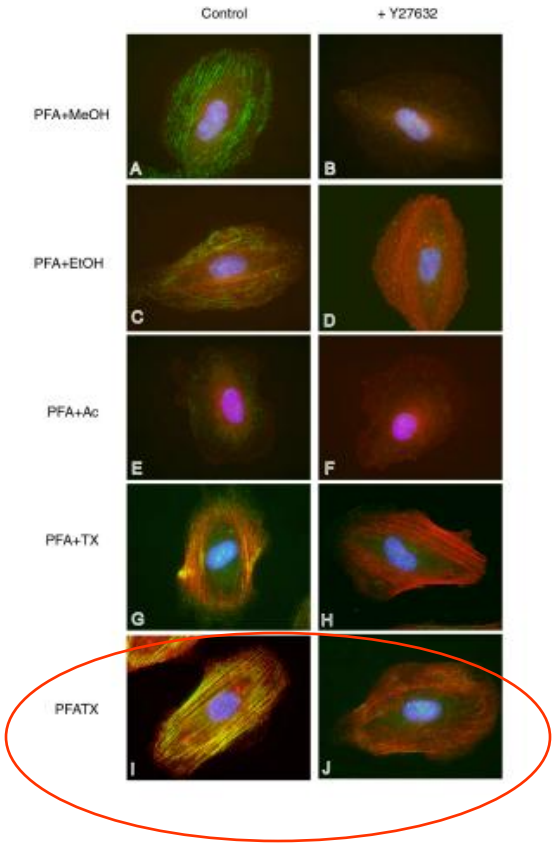
Cell cycle times: 17.1 h +/- 4.3 h (man); 17.3 h +/- 4.3 h

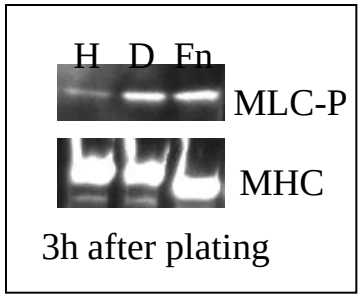
Halter et al. *Cytometry A*, 2011

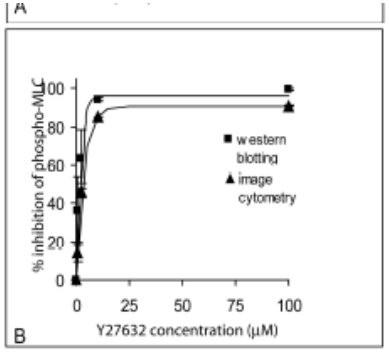
Validating Cell Imaging Protocols

Effects of Fixation

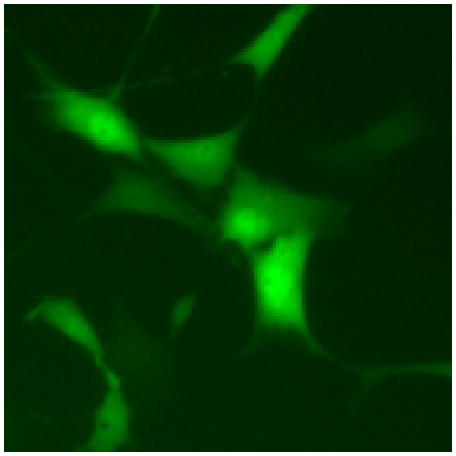
Phospho-myosin



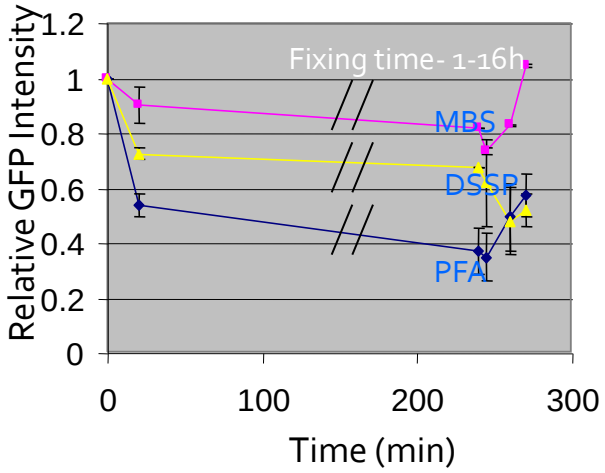




Cytoplasmic GFP



In situ cell-by-cell analysis



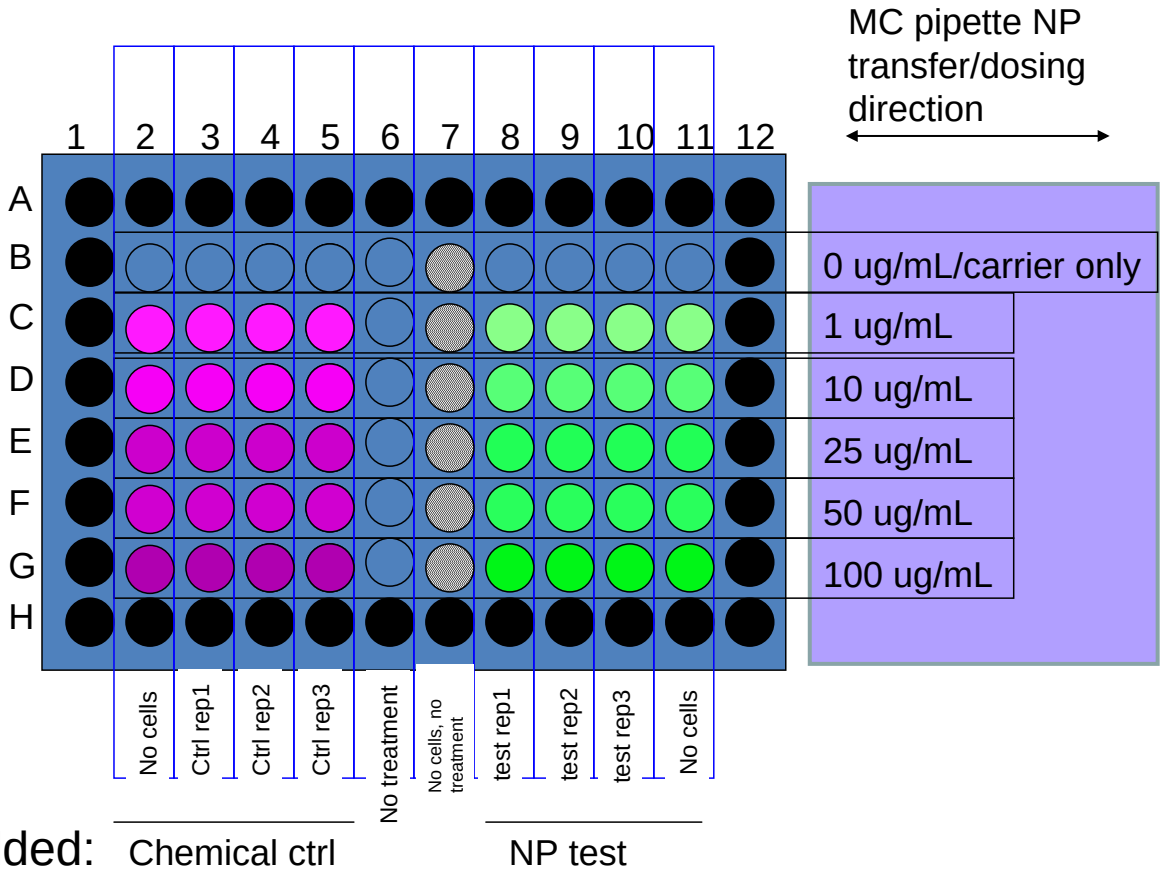
Bhadriraju et al BMC-Cell Biology 2007

Langenbach et al Biointerphases 2006

Redesigning Cell-based Assays with Validation Criteria: Nanocytotox

International Alliance for NanoEHS Harmonization

•Stage 3.1 (2010)- Lack of agreement in interlaboratory testing (9 expert academic labs)
•**Why??**



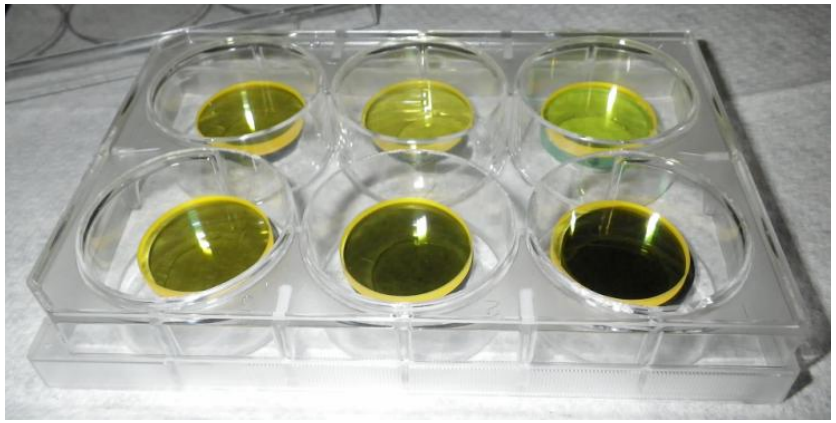
•Several control experiments added:
- -within pipette variability
- -between pipette step variability
- -collection of raw absorption data
- -no cell NP test

•Protocol modified:
- -directed pipette procedure
- -directed dilution procedure
- -directed NP dispersion procedure

Validation criteria:
- -within pipette variability <6%
- -between pipette step variability <10%
- -OD <1.2 in no treatment wells
- -reagent only well variability <3%
- -EC50 CdCl2 ctrl ~30 uM
- -no absorption in NP only wells

Instrumentation Benchmarking Tools

Fluorescence Imaging System

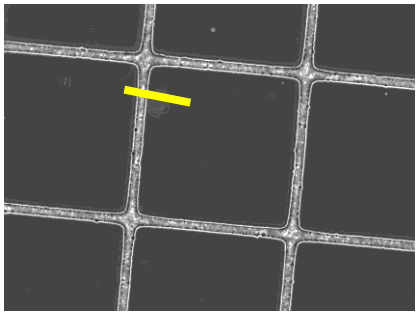


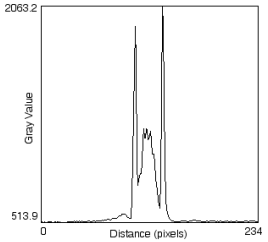
Prototype fluorescent microscope benchmarking tool- Halter 2012

- Photostable fluorescent glass
- Dynamic range
- Signal to noise
- Lower limit of detection
- Upper limit of detection (saturation)

Phase Microscopy

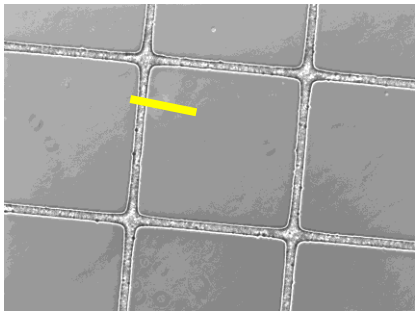
Correct alignment

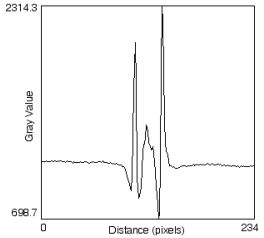




PDMS stamp on glass

Incorrect alignment





Halter et al 2011

- Need for benchmarking tools and protocols to specify the quality of an imaging system

Summary

- Need quantitative measurements to assess the quality of an assay method
  - Accuracy, precision, robustness, reproducibility
- Requires reference data, reference materials (standards), benchmarks, controls, statistics
- Experimental design can be used to establish robustness to factors
  - Fabrication, culture conditions, imaging system, etc.
- Cell imaging requires validation of image analysis, cell preparation protocols and benchmarking of imaging conditions
- Specifications that must be met before an assay is deemed valid can be identified with experimental design and quantitative measurements.