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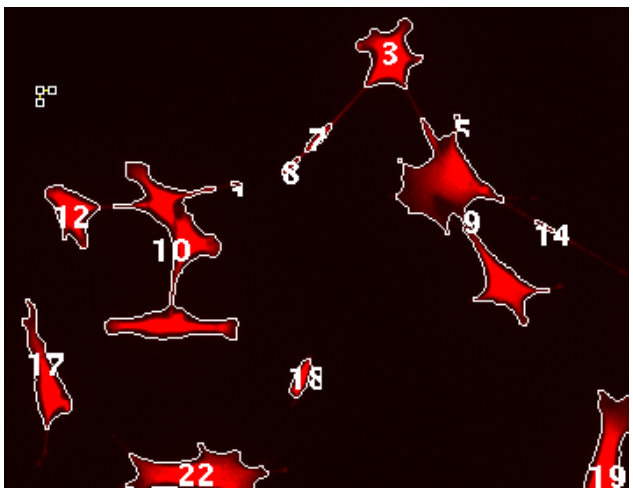
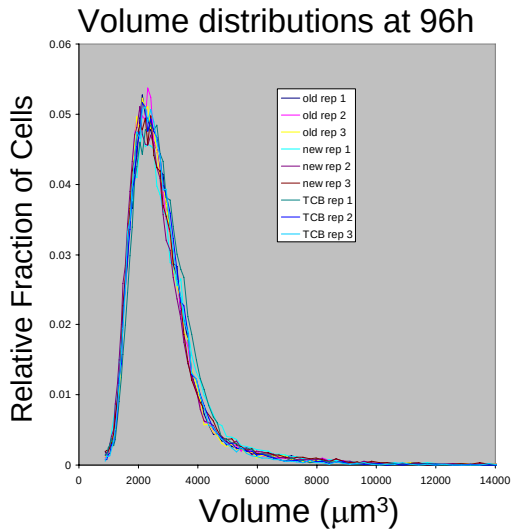
Considerations for Quality Control of In Vitro Cell Cultures





John T. Elliott (NIST), Alex Tona (NIST), Michael Halter (NIST), Steve Bauer (CBER/FDA), Abeba Tesfaye (CBER/FDA), and Anne L. Plant (NIST)
NIST, Cell and Tissue Measurements Group, Gaithersburg, MD 20899

Cell Measurements from the NIST Point of View

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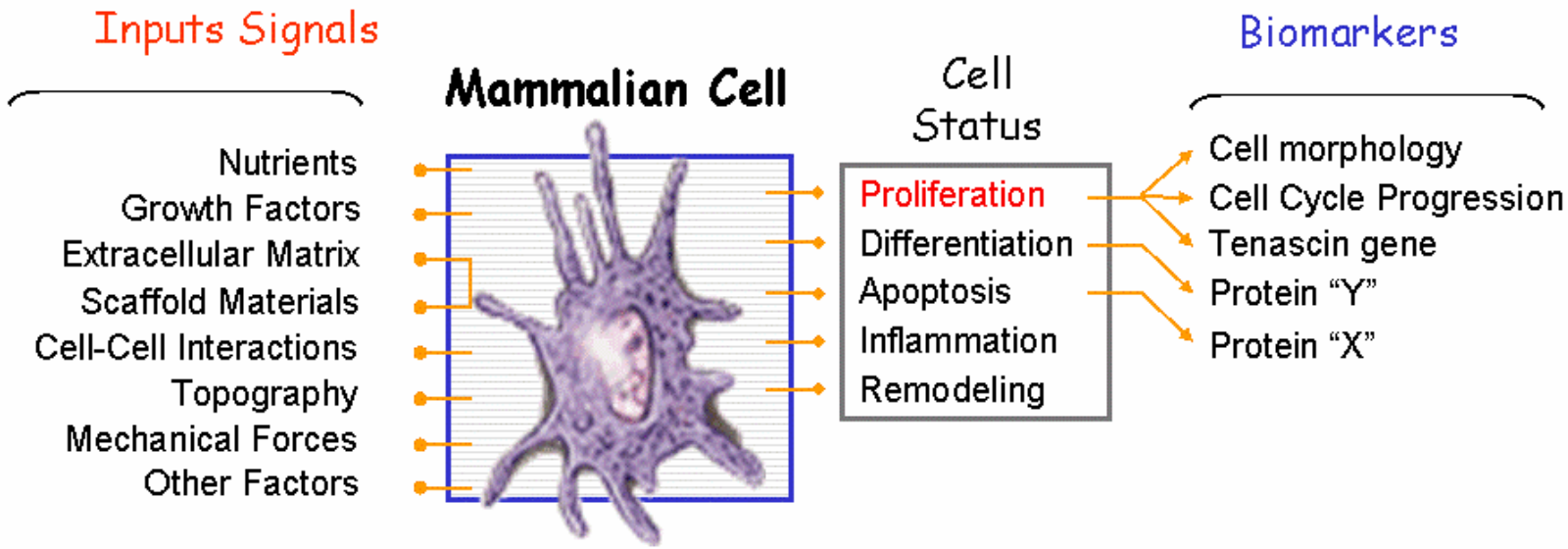
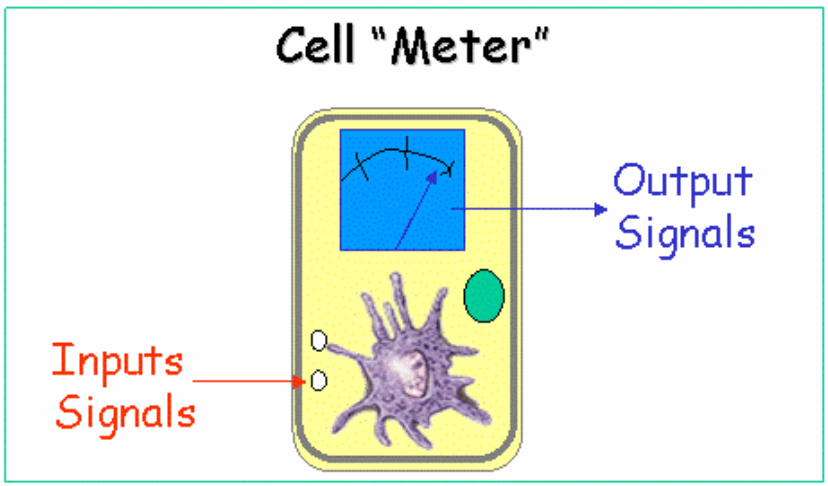
- **NIST's mission:** To promote U.S. innovation and industrial competitiveness by advancing measurement science, standards, and technology …. **Non-regulatory.**

- Facilitate Measurements
  - Consensus standards, standard reference materials
- New measurement techniques
  - Application of advanced physical and chemical measurement to biology
- Extracting new information from existing data
  - Application of modeling and statistical theory to biological data

- We focus on the measurement infrastructure
  - How robust is this measurement?
  - Is this high quality data?
  - What is the best way to represent the data?
  - Does every laboratories get the same answer?
  - What is the best statistical technique for detecting differences?

Using Cells as Measurement Devices





How do we know the meter is functioning correctly?

Quality Control in Cell Culture

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Validation Question: Are the cells behaving as expected before we use them?

Cell culture is the process of keeping cells alive under *ex-vivo/in vitro* conditions:

- -Expanding cell number
- -Cellular assays

Ex-vivo/In vitro conditions (i.e. artificial environment):

- -Incubators, CO_2, pH, extracellular matrix, TCPS, nutrients, hormones, passaging/trypsinization, freezing/thawing

Do we have specifications to ensure the nominal behavior of cells in culture?

Identifying General Quality Control Metrics for Cell Culture

NIST POV: Which cell measurements are good candidates for quality control metrics?

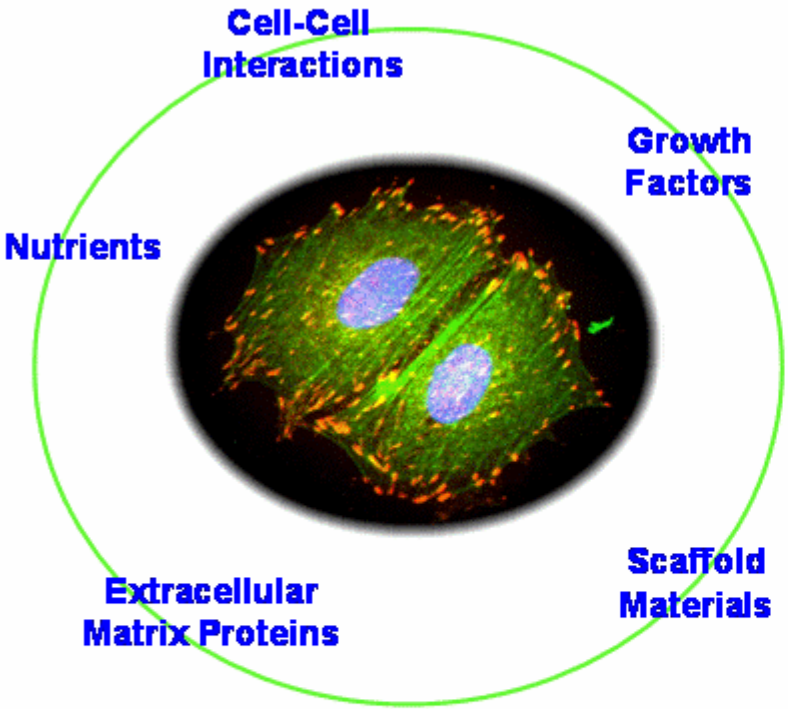
- -Robust and Routine
- -Calibrated/Traceable
- -Measurement linked to cell processes
- -Generates high quality data

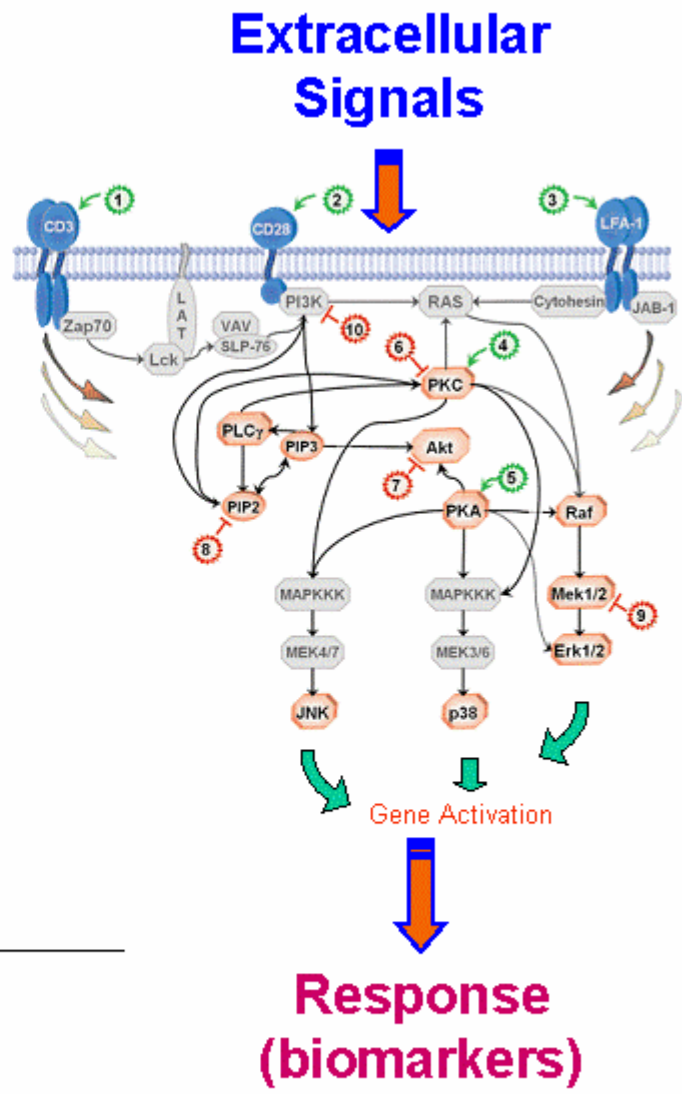
Two candidates of interest:

- -Cell Volume Measurements
- -Cell Spreading/Morphology Measurements

Origin of a cellular response

Extracellular signals include:





Measurement	Signaling Pathways
Cell volume	cell cycle, cell growth
Cell spreading	cell cycle, cell growth, cell adhesion

Expect a distribution of cell responses

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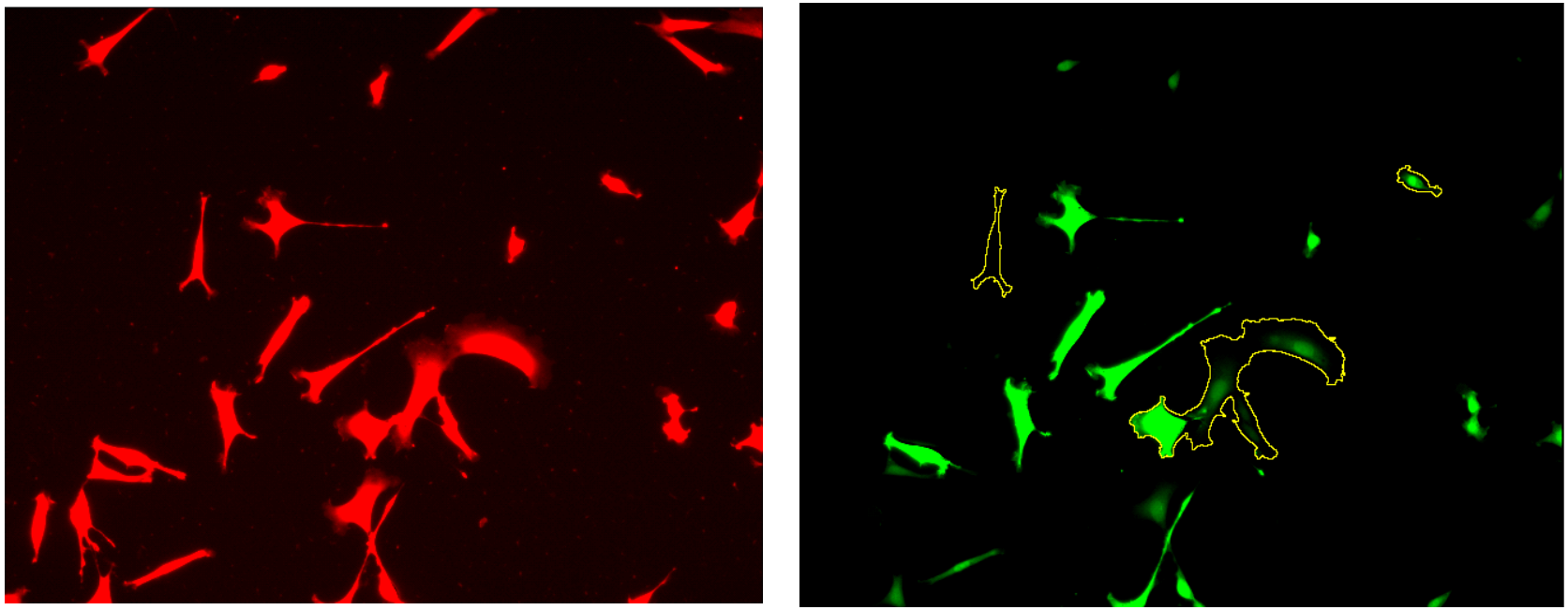
Cell Shape

Gene Activation (TN1-GFP)

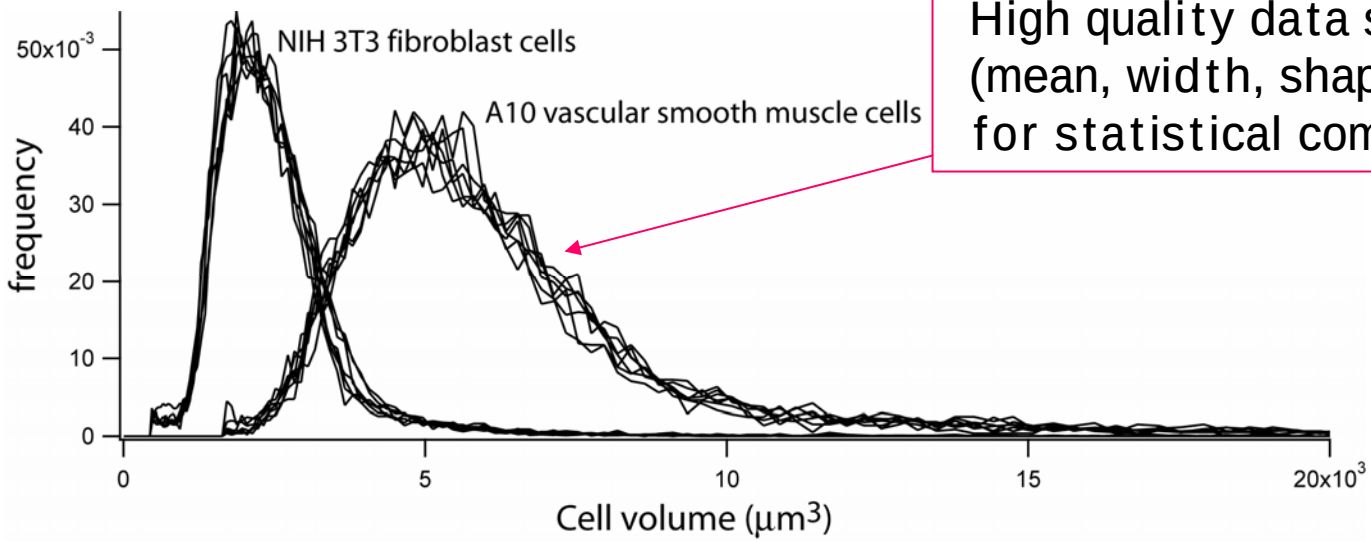


Single cell clone of NIH3T3-TN1-GFP-fibroblast on TCPS

- Information can be obtained from the distribution of the data.
- Obtaining distribution data requires single cell measurement techniques

Cell Volume Measurements

-Electronic cell volume measurements have been around for 50 years.
-Provides data describing the distribution of cell volumes.
-This distribution appears to be stable in expanding cells
-Demonstrates differences between cell lines.



High quality data sets (mean, width, shape). Good for statistical comparisons.

Multiple passages of a vSMC and fibroblasts

Origin of the Cell Volume Distribution

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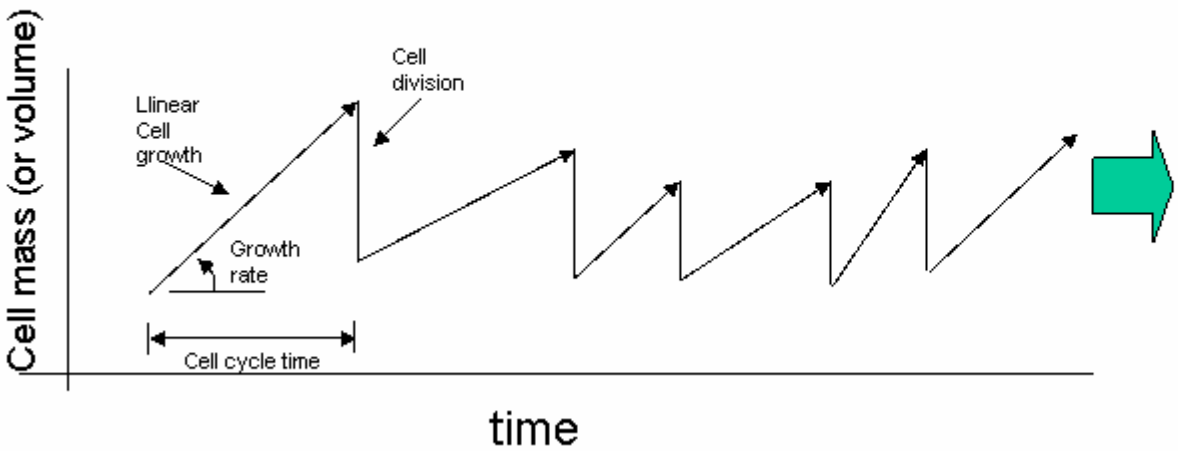
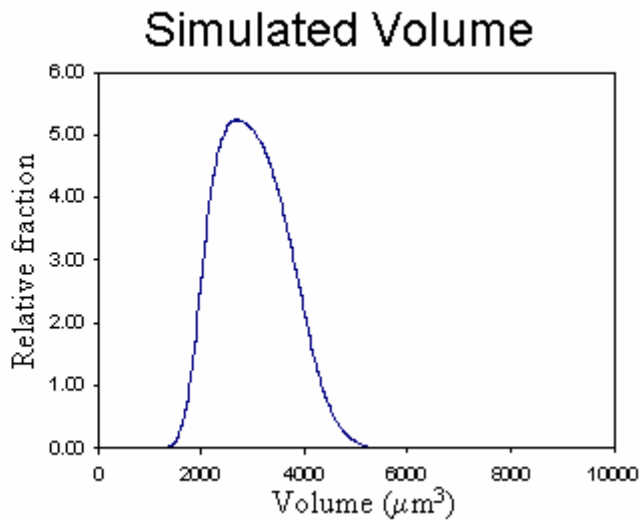
- Assume the density (mass/volume) of a cell is constant through cycle.
- Cell mass is regulated by total expression of house keeping genes

- Assume cell divides exactly in half during division
- Assume cell cycle time and cell growth rate are picked from a normal distribution.
- Simulate growth and division of a cell population





Predictions using the Cell Volume Distribution Model

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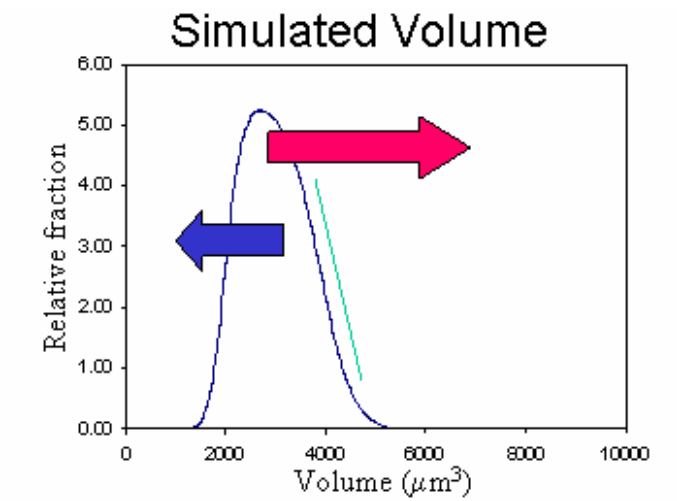
Shift Right

increase growth rate
increase cell cycle time

Shift Left

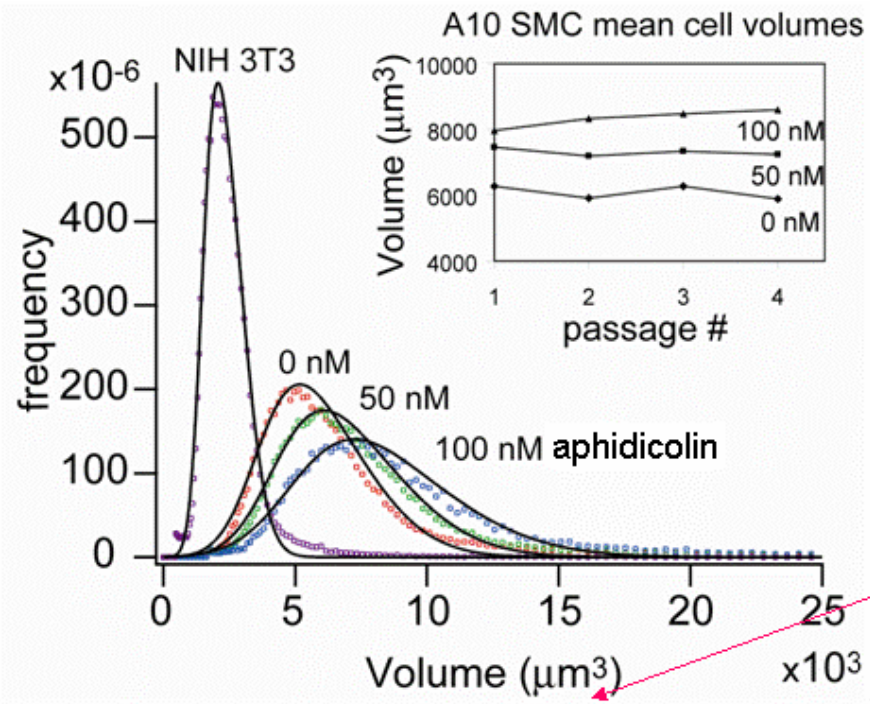
decrease growth rate
decrease cell cycle time

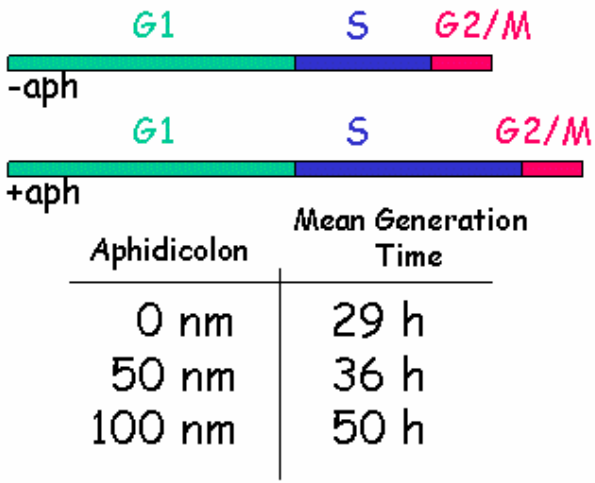
Changes in noise will influence shape parameters



By using this model we can understand the measured cell response

Testing the model





Aphidicolon	Mean Generation Time
0 nm	29 h
50 nm	36 h
100 nm	50 h

We can define the distribution in terms of mean cell cycle time, mean growth rate and variance in these parameters.

Table 1. Parameter values estimated from Coulter counter data.

Cell type[a]	t^b (h)	σ_t^c (h)	r^d (µm³/h)	σ_r^e (µm³/h)	$\left(\frac{\sigma_t}{t}\right)^2+\left(\frac{\sigma_r}{r}\right)^2$	μ_N[g] (µm³)	σ_N[h] (µm³)	$\left(\frac{\sigma_N}{\mu_N}\right)^2$
NIH 3T3	19.5	3.9	79 ± 0.3	28.2 ± 0.6	0.17	2,272	651	0.08
A10 (0 nM)	29	5.8	133 ± 1	58.9 ± 1.6	0.24	6,053	2,105	0.12
A10 (50 nM)	36	7.2	126 ± 1	55.9 ± 1.3	0.24	6,909	2,274	0.11
A10 (100 nM)	50	10	108 ± 1	52.1 ± 1.3	0.27	8,191	2,837	0.12

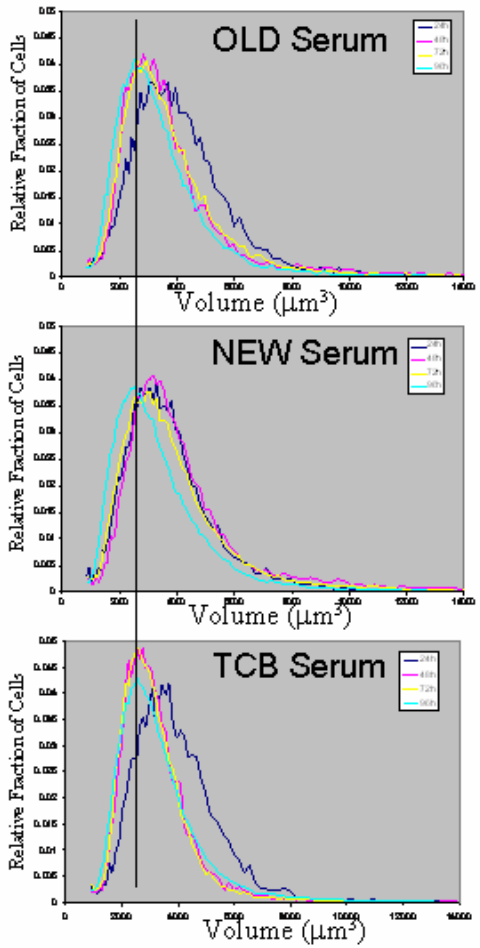
[a] A10 SMC's were continuously cultured with three different concentrations of aphidicolin, 0nM, 50nM, and 100nM.
[b] Cell cycle times (mean generation times) estimated by counting cells over f passages.
[c] Cell cycle time variability estimates were $0.20 \times t$.

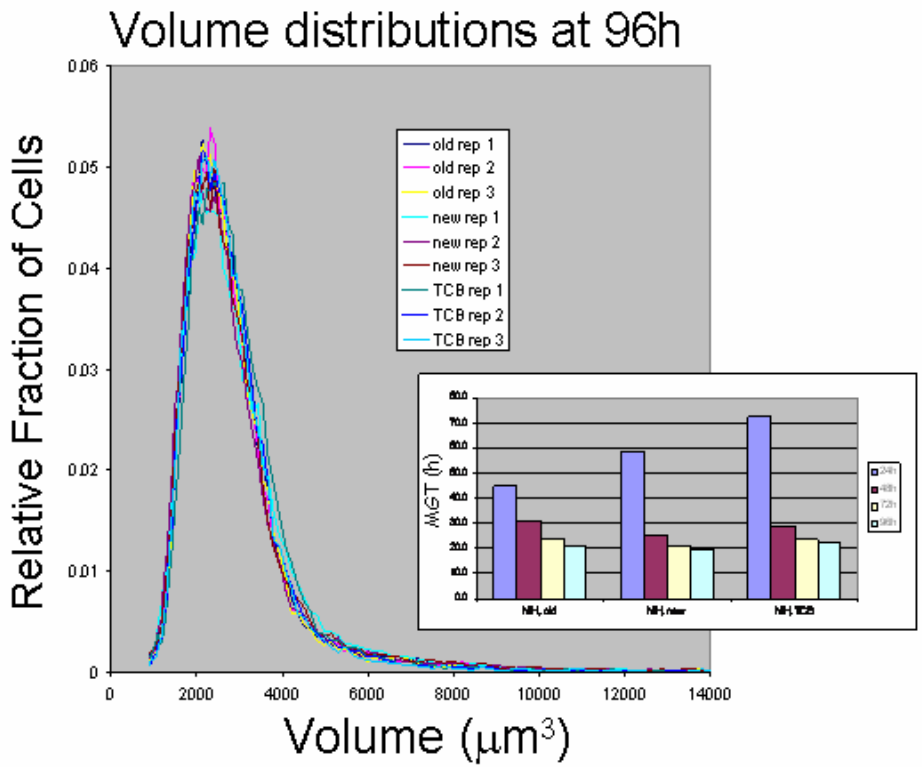
$$f(V') = \frac{1}{t}\cdot\int_{\tau}^{\infty}\left(\frac{1}{\sqrt{\frac{1}{3}(\tau\cdot\sigma_t)^2+\frac{1}{3}(\tau\cdot\sigma_r)^2}\cdot\sqrt{2\pi}}\cdot e^{\frac{-(V'-\tau\cdot t)^2}{2\left(\frac{1}{3}(\tau\cdot\sigma_t)^2+\frac{1}{3}(\tau\cdot\sigma_r)^2\right)}}\right)\cdot\left(\frac{1}{2}-\frac{1}{2}\,erf\left(\frac{\tau-2\cdot t}{\sqrt{2}\cdot\sigma_t}\right)\right)d\tau$$

M. Halter et al. in preparation.

Example: Effect of Passage/Serum

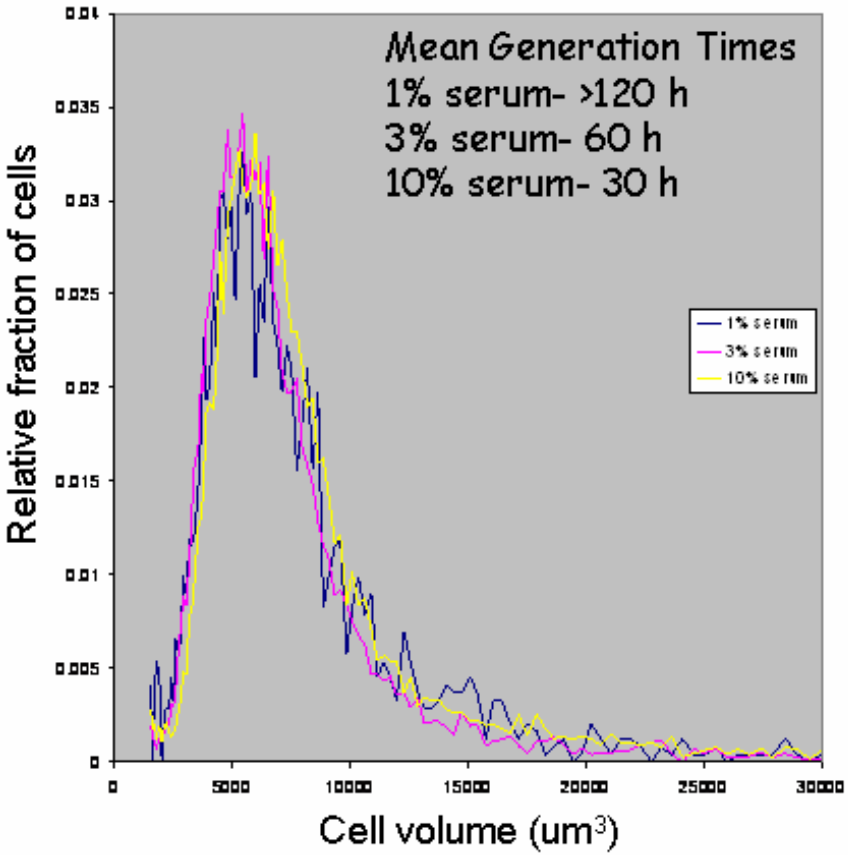
Volume Distribution of NIH 3T3 cells over 96 h after replating





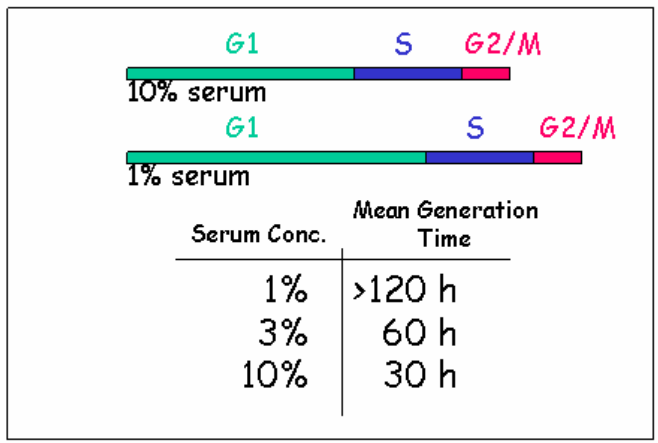
- Data suggests that after 96h, cell cycle time and growth rates are independent of serum.
- Serum type does appear to influence lag time after plating.

Example: Varying Serum Concentration



-A10 cells exhibit a stable volume distribution in significantly reduced serum:

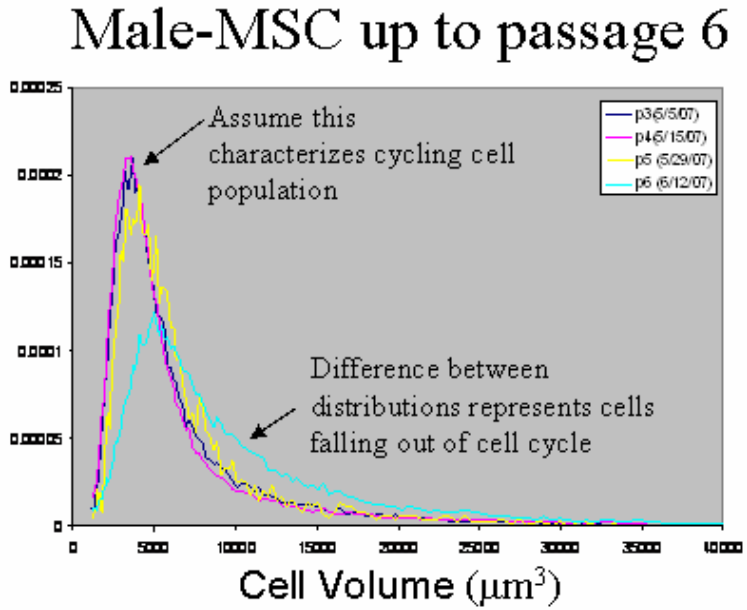
-MGT goes up, but volume stays the same. This indicates the growth rate decreased.

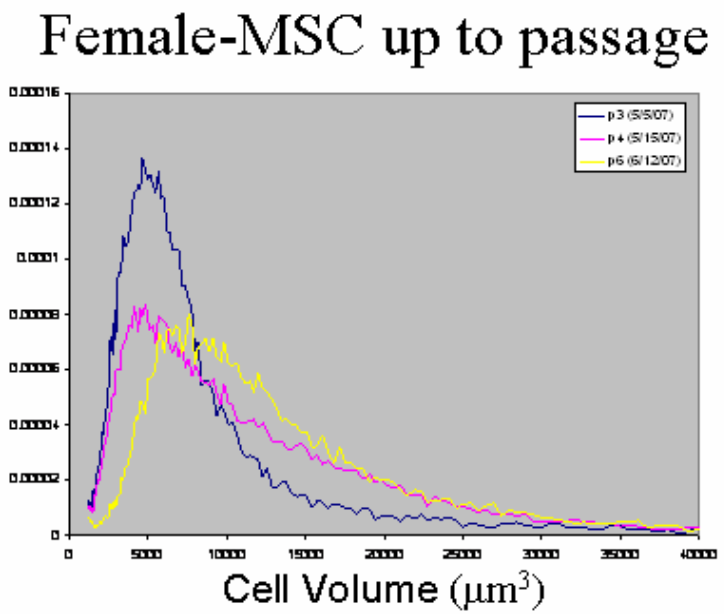


Serum Conc.	Mean Generation Time
1%	>120 h
3%	60 h
10%	30 h

-Use of the cell volume distribution model provides additional information about the cell culture.

Example: Male and Female MSCs in culture-volume measurements 1





- This result indicates that in this experiment, the male MSCs were stable for several passages before shifting in cell size begins.
- Volume measurements can provide information on a population of cells with increasing heterogeneity.
- Using a model allows us to understand what is happening.

Mesenchymal Stem Cells Morphology

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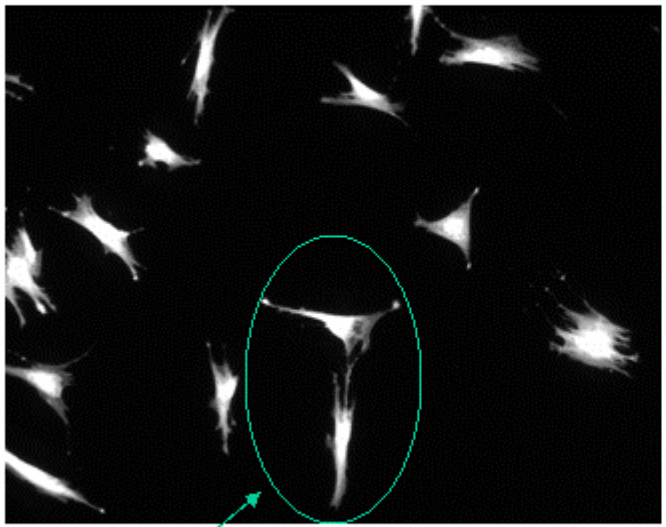
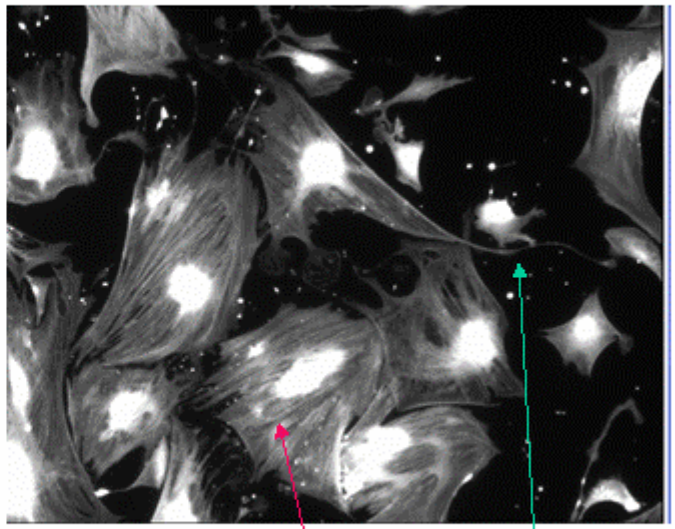
Seeded at 50 cells/cm^2, passage 3



Proliferating cells

Seeded at 3000 cells/cm^2, passage 8



Senescent cells

Proliferating cells

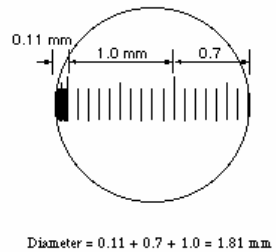
- In collaboration with FDA (Steve Bauer, CBER), we are using morphology to measure stability of MSC cultures
- Cell volume is proportional to its spreading area.

Thinking about Cell Morphology Measurements

- Quantitative and traceable measurement

  -Standards for spatial calibration of microscope available

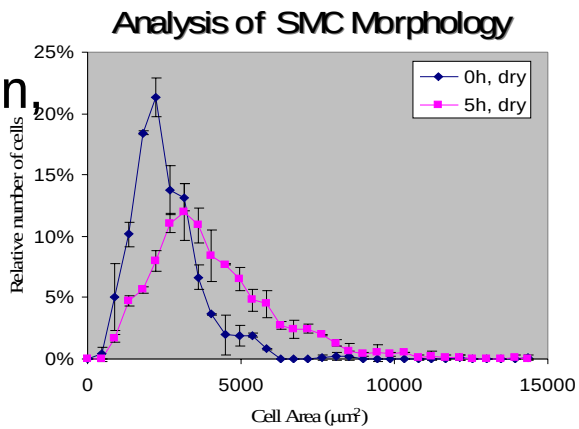
  -Facilitates intra-laboratory comparisons



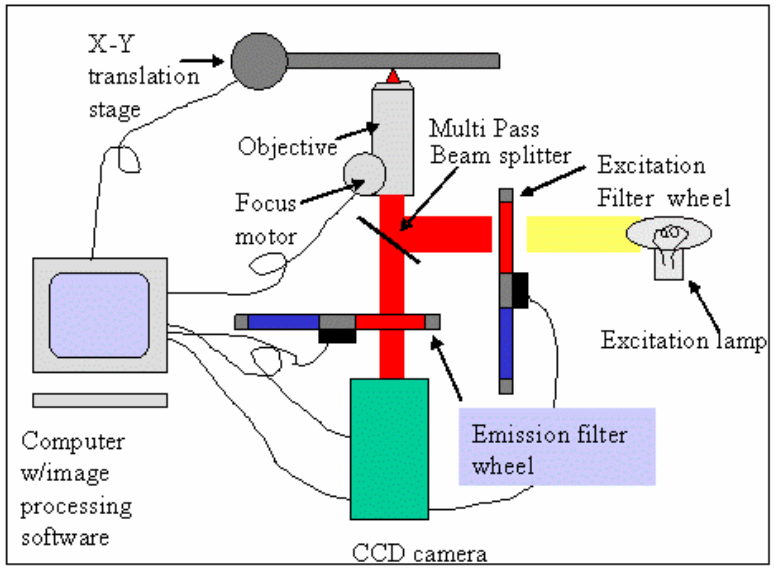
- Cell-by-cell measurement technique

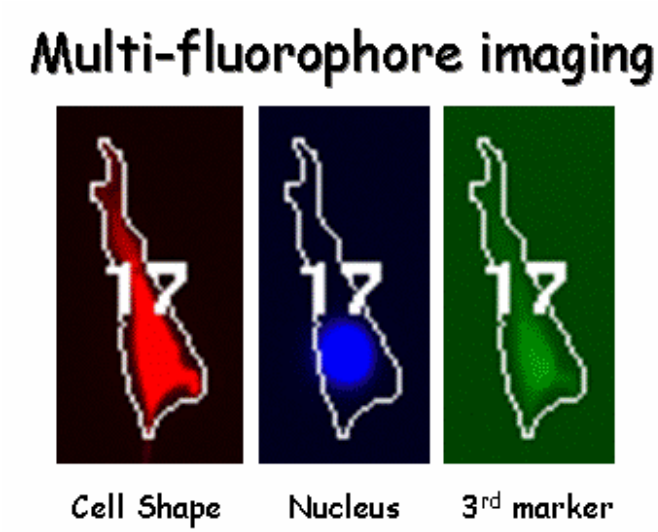
  -Measure the distribution of cell morphologies

  -Cell morphology is linked to cell adhesion, cell cycle, cell growth and cytoskeletal pathways.



Automated Quantitative Microscopy

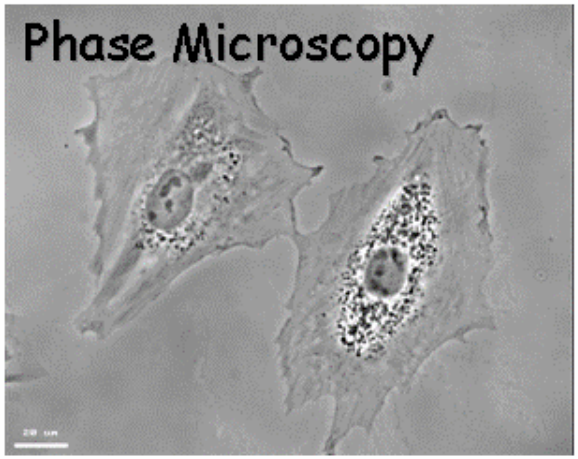


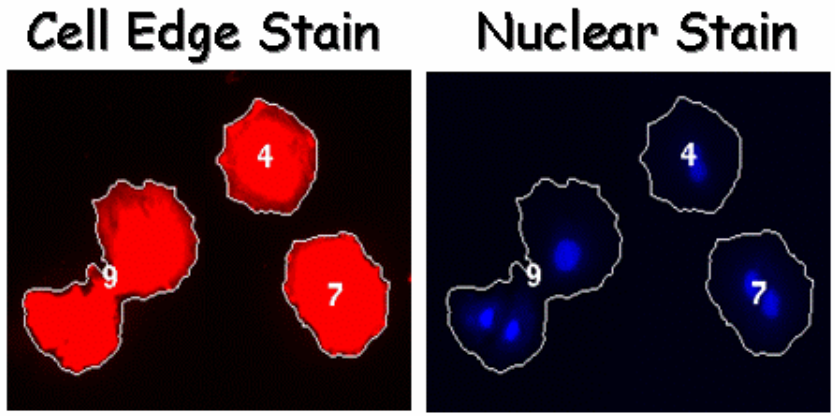


Advantages:

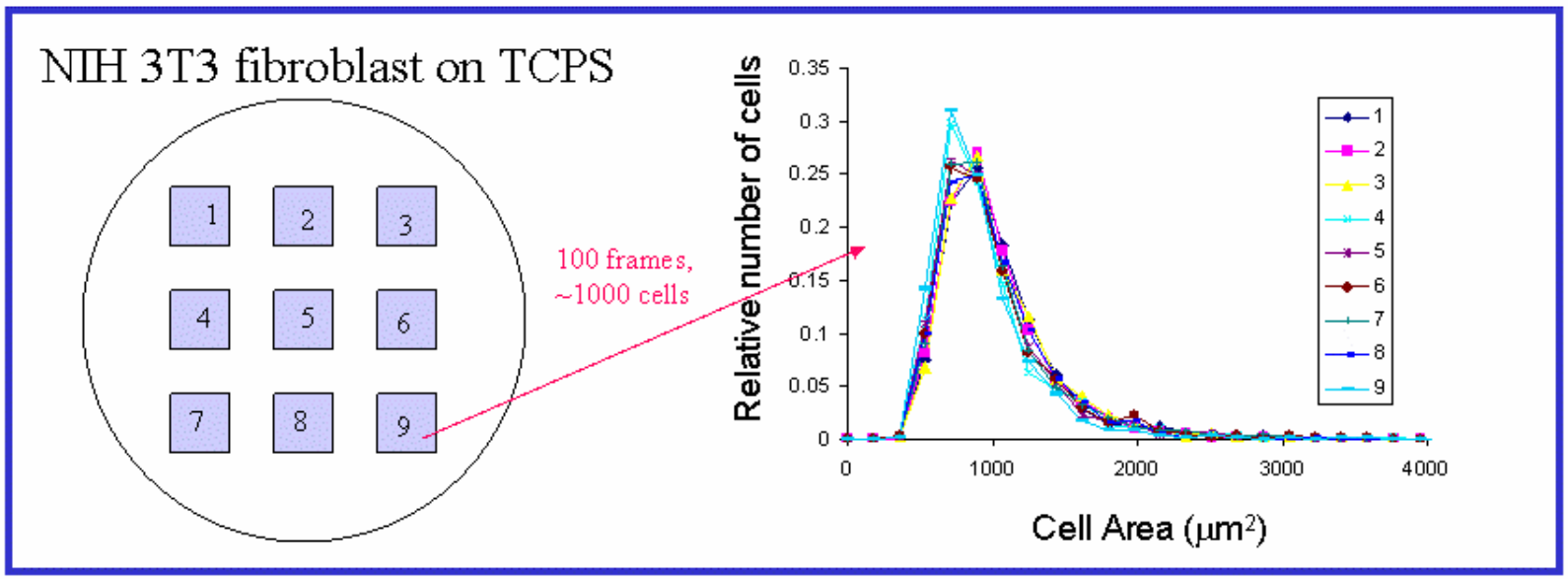
- -Unbiased data collection
- -Sample large number of cells
- -Multi-fluorophore imaging
- -Live cell imaging
- -Evaluate cells in real culture conditions

Measuring Cell Morphology



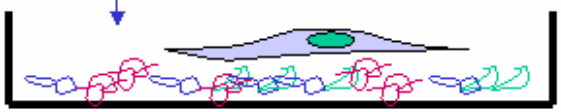


Elliott, et al. Cytometry 2003, Langmuir 2003



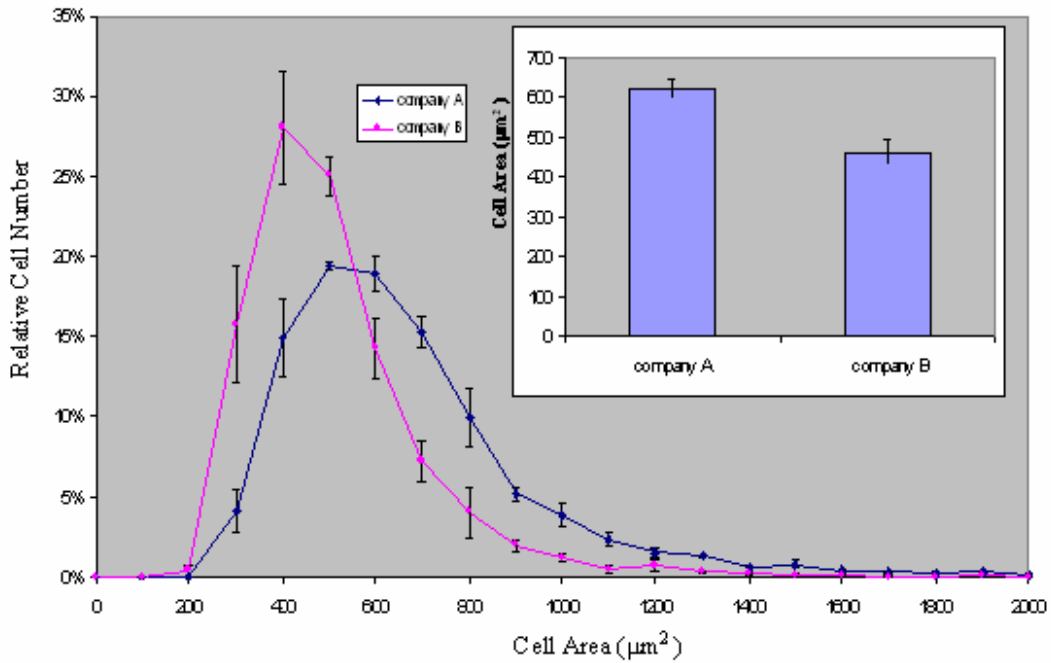
Morphology depends on Substrate

- Serum proteins
  - -vitronectin
  - -albumin
  - -others
- Specific pro-adhesive proteins



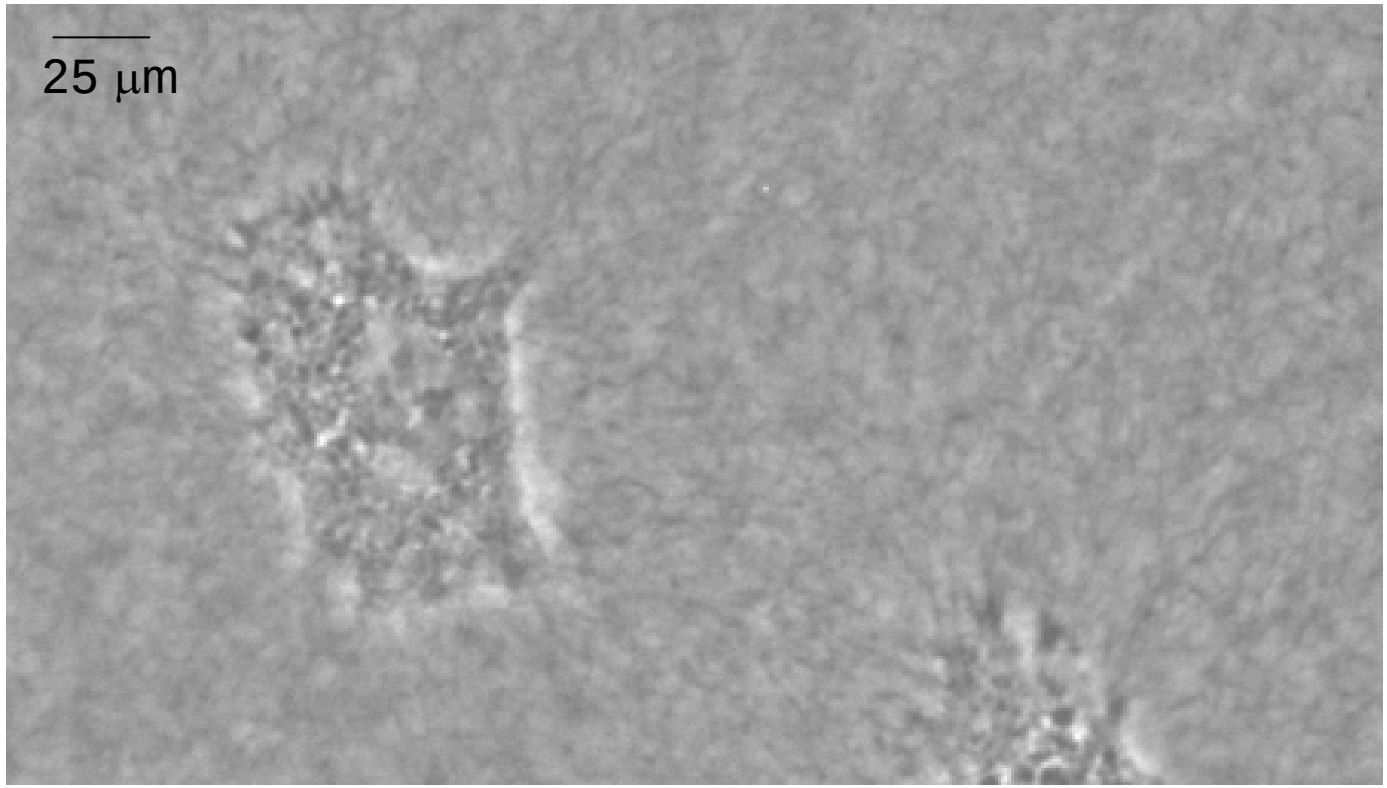
Tissue Culture Polystryene

Protein presentation
- -specificity of adsorption
- -conformation/structure
- -surface chemistry



Variations in NIH-3T3 cell morphology on TCPS from different manufacturers is likely due to differences in adsorbed protein.

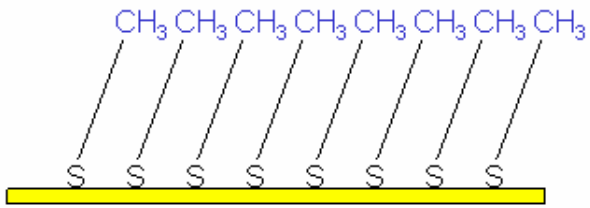
Collagen Gels in Experiments



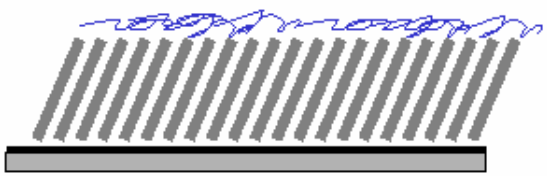
- No standard methods for preparing extracellular matrix.
- Difficult substrates for microscopy.
- Native collagen gels are very fragile (big problem!).
- Very difficult to systematically control the physiochemical properties of collagen fibrils

Preparation of Collagen Thin Films on Alkanethiol Monolayers

- Alkanethiol ($C_{16}SH$) self-assembled monolayer on translucent Au



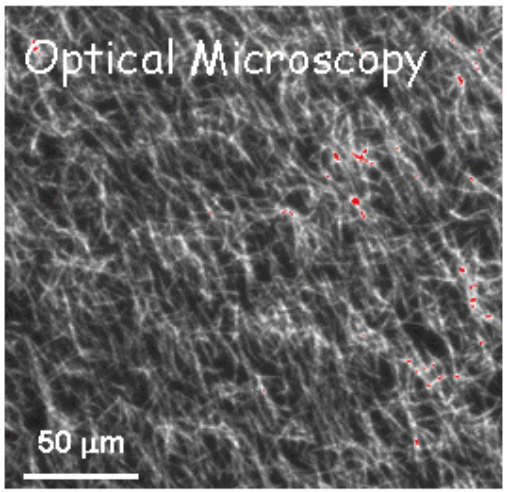
1. Incubate with native collagen, 37°C, neutral pH
2. Rinse well
3. Blow dry
4. Rehydrate

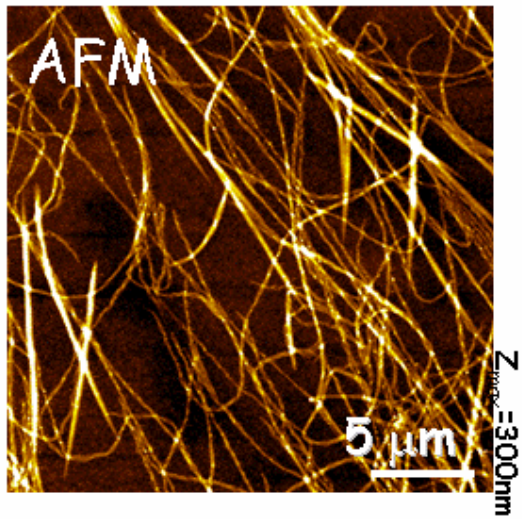


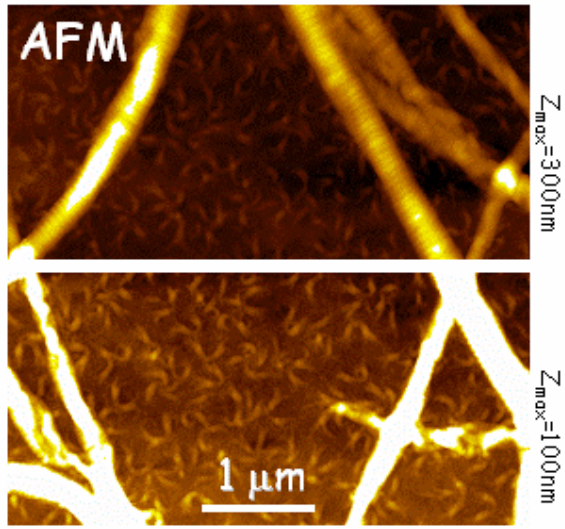
Advantages of thin protein films on alkanethiol monolayers:

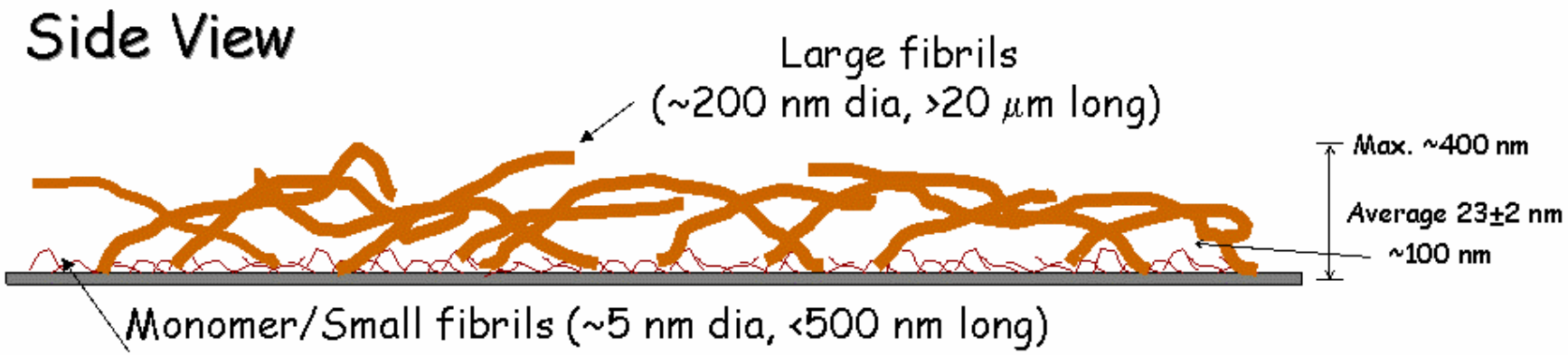
1. Highly reproducible and homogeneous starting surface.
2. Can be characterized and verified with surface analysis techniques.
3. Very robust and easy to use.
4. Excellent microscopy properties.

Native Fibrillar Collagen Thin Films

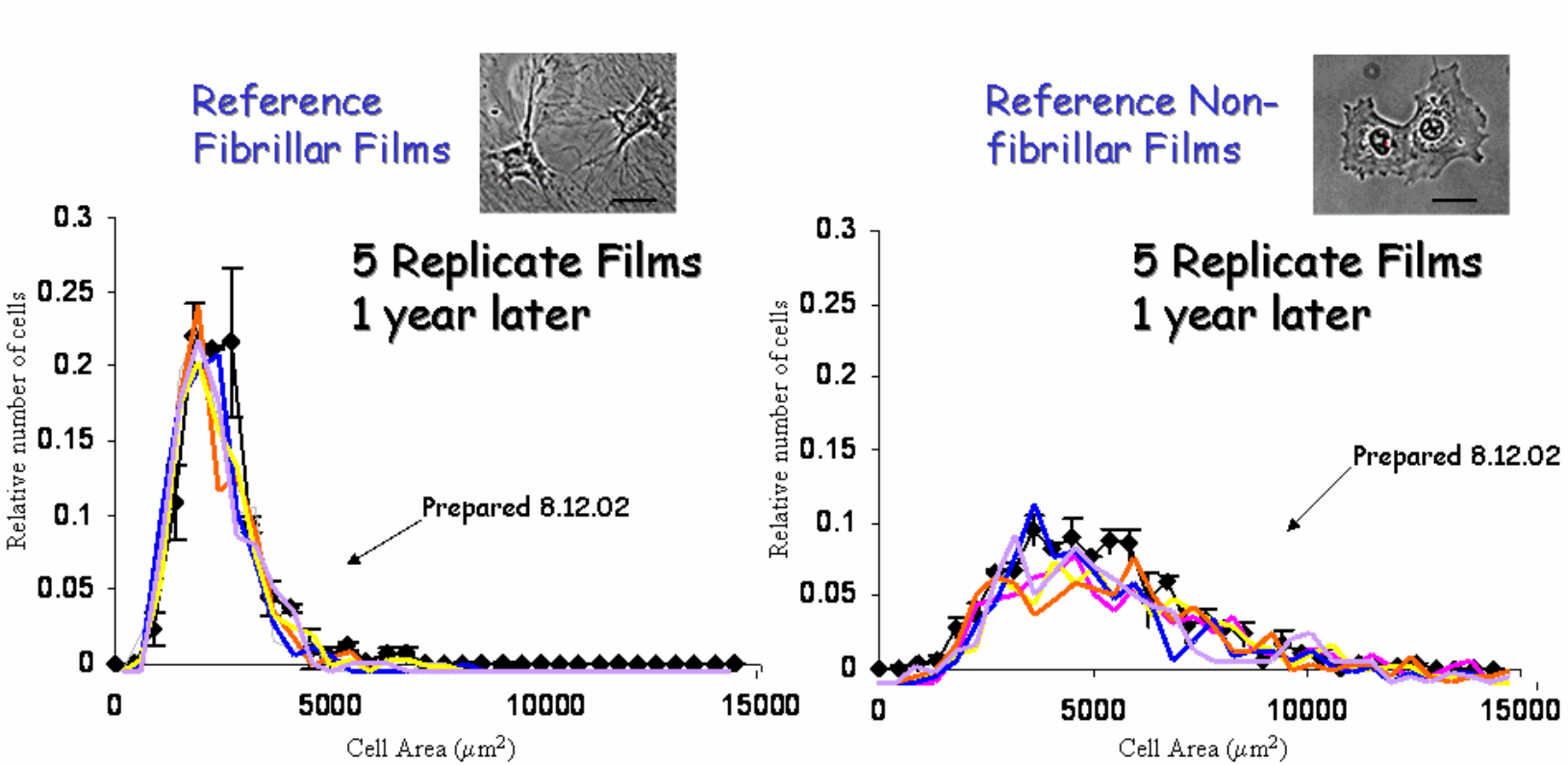






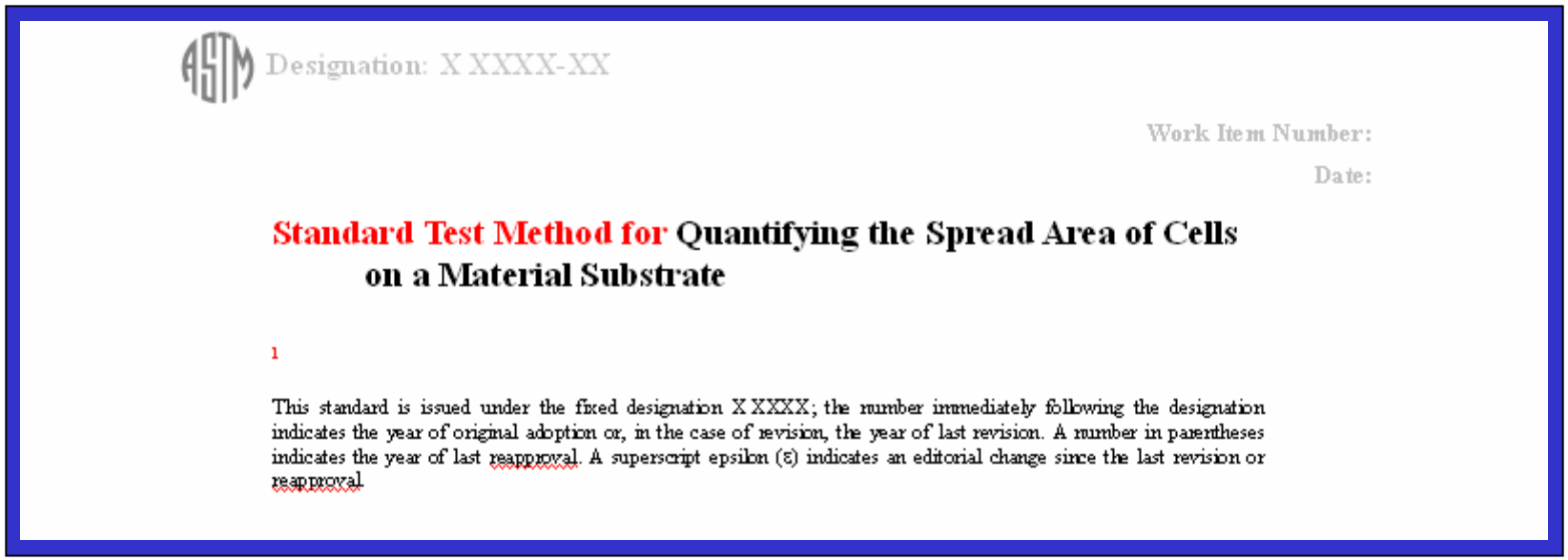


Morphology Metrics from Cells on Reference Materials



- Integrin engagement pathways, cell volume, etc appear to be nominal.
- We can think about quality control metrics for cell culture

ASTM Standard For Measuring Cell Morphology



Designation: X XXXX-XX

Work Item Number:

Date:

Standard Test Method for Quantifying the Spread Area of Cells on a Material Substrate

1

This standard is issued under the fixed designation X XXXX; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

- In 2007, we initiated a Standard Test Method document for ASTM F04 Medical and Surgical Materials and Devices (F04.46- Cell Signaling Subsection)

- Provides a SOP for cell morphology measurements
- Think about standards you need!!!

ASTM Std for Morphology Details:

- Selection of substrate/material
- Adhesion time
- Volume measurement
- 2-color image collection
- 2-color image analysis
- Uncertainty Issues

- Not answered is how to make it a specificaton

Conclusions

-Important to think about cell morphology measurements in terms of cell processes/signaling pathways

-Single cell measurement techniques provide information about the population of cells

-Cell volume and cell morphology measurements are interesting metrics because standards to facilitate intra-laboratory comparisons of data exist.

-Cell volume distributions provide insight on the cell cycle and cell growth properties of a culture.

-Cell morphology can provide insight on the status of adhesion signaling pathways in a cell culture.

-These measurements can be used as quality control measurements, but it is important to understand what influences the measurement.