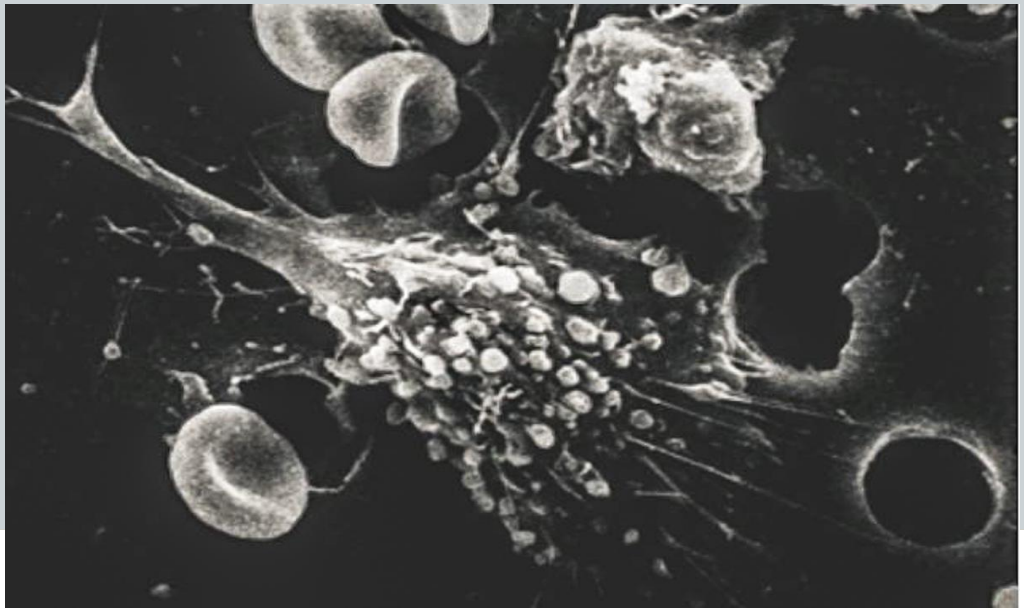
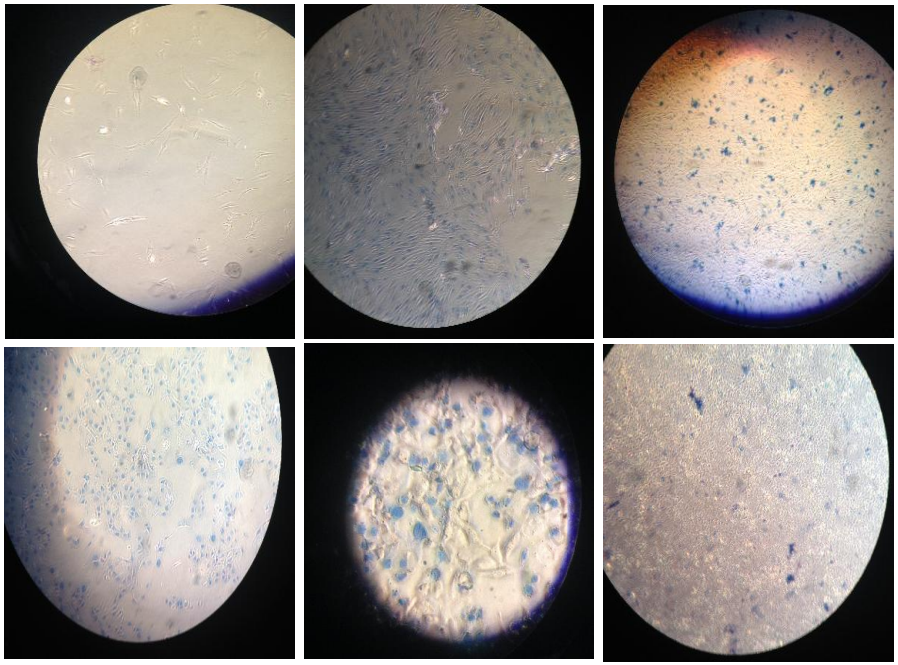


CYTOTOXICITY

Other Agenda: Reporting your Trypan Blue And Growth Curve Data



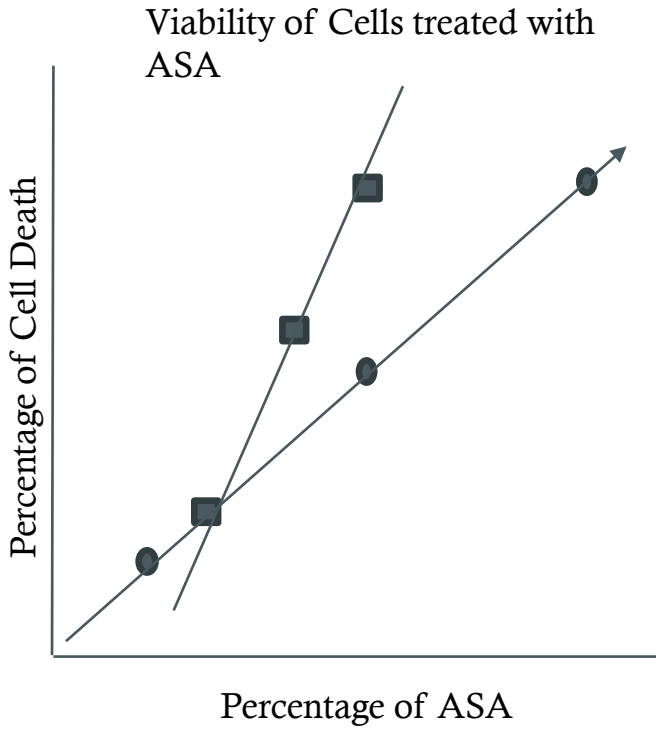
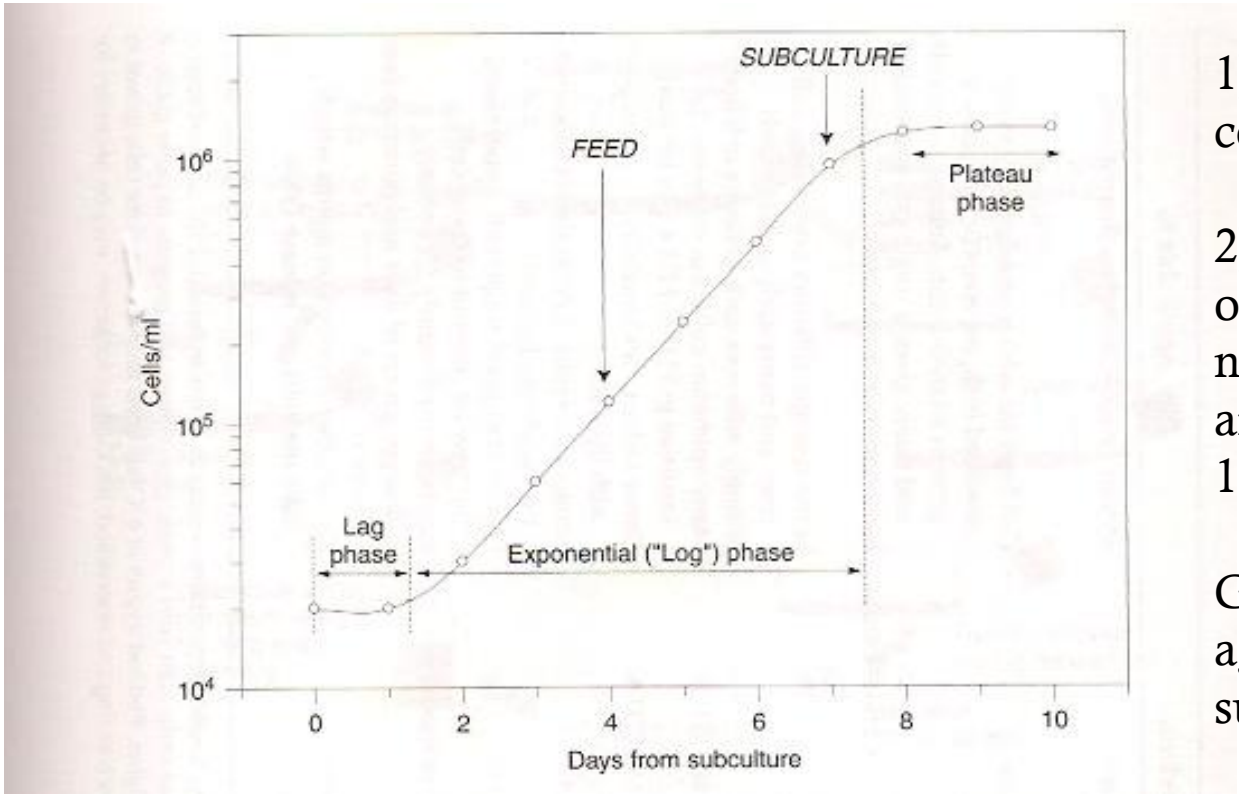


Figure 1. Trypan Blue experiment to find the IC50 of ASA for HFF and U2-OS cells. **A.** HFF cells exposed to 10% ASA. **B.** HFF cells exposed to 100% ASA. **C.** HFF cells exposed to 50% ASA. Approximately 50% of the cells were observed as undergoing cell death. **D.** U2-OS cells exposed to 15% ASA. **E.** U2-OS cells exposed to 35% ASA. Approximately 50% of the cells were undergoing cell death at that concentration. **F.** U2-OS cells exposed to 50% ASA.

GROWTH CURVE



1) Estimate percentage of confluence in image.

2) Convert the percentage of confluence to cell number. Assume there are 2.6 x 10^6 cells in a 100% confluent flask.

Graph Cell number against Days from subculture.

Cytotoxicity

Def: *(n.)* The degree to which an agent possesses a specific destructive action on certain cells

Cytotoxicity Assay

Def: *(n.)* An investigative procedure in laboratory science to determine the toxic quality of an agent to cells

APPLICATIONS

- Safety of pharmaceuticals
- Safety of cosmetics
- Safety of food additives
- Anticancer agent drug screening
- Predictive drug testing for tumors







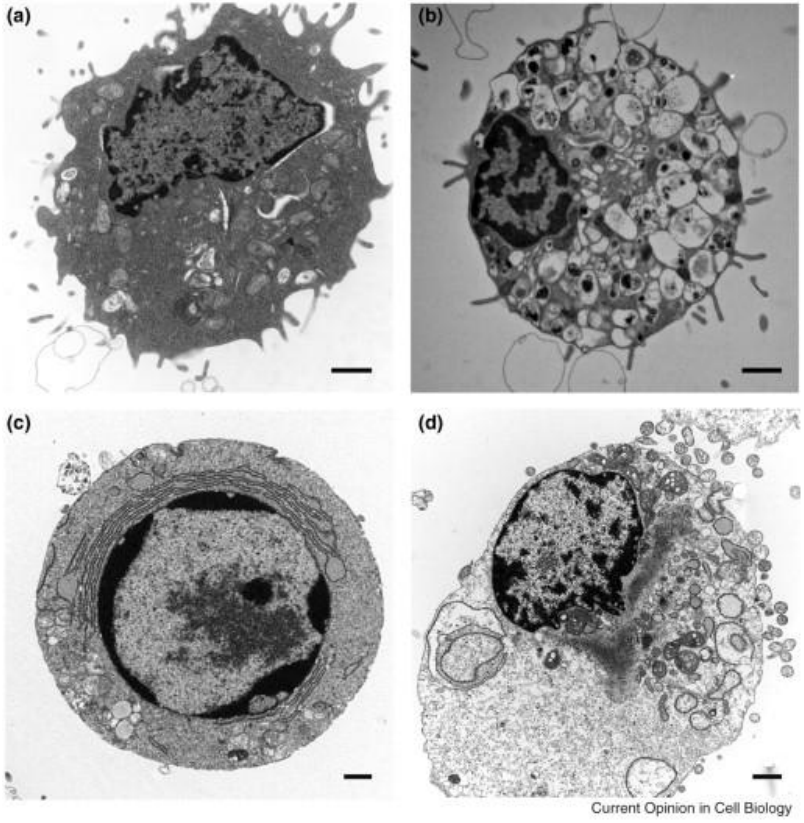


Toxicity is a complex event with many definitions:

- direct cellular damage
  Eg. disruption of membrane integrity
- disruption of cellular metabolism
  Eg. REDOX reactions
- physiologic effects
  Eg. disruption of membrane transport in the kidneys
- systemic effects
  Eg. nausea, pyrexia, hypotension, etc
- inflammation effects
  Eg. allergic reactions

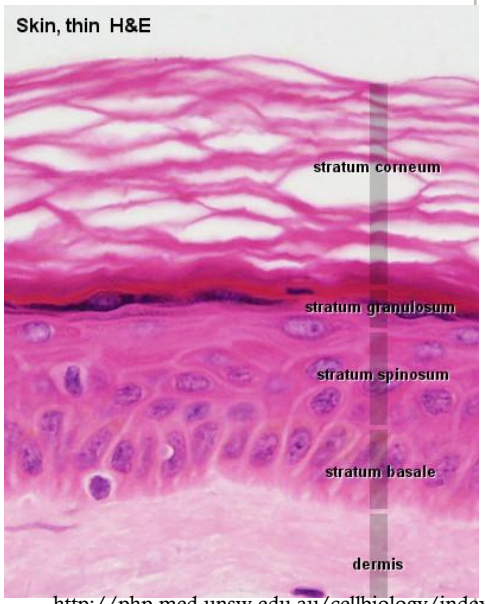
Even demonstrating cell death (a *cytocidal* effect) is not straight-forward:

Edinger, Thompson (2004) *Death by design: apoptosis, necrosis and autophagy*



apoptosis

Terminal differentiation (e.g. cornification)

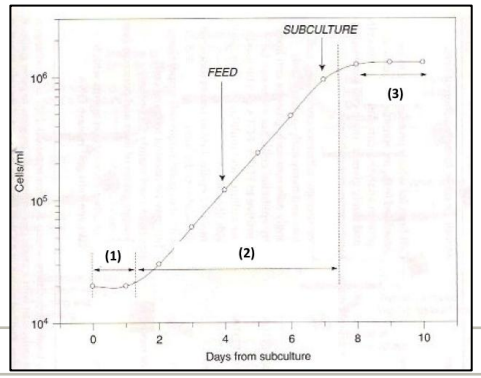


http://php.med.unsw.edu.au/cellbiology/index.php?title=Cell_Death_1

Self-digestion (*autophagy*)

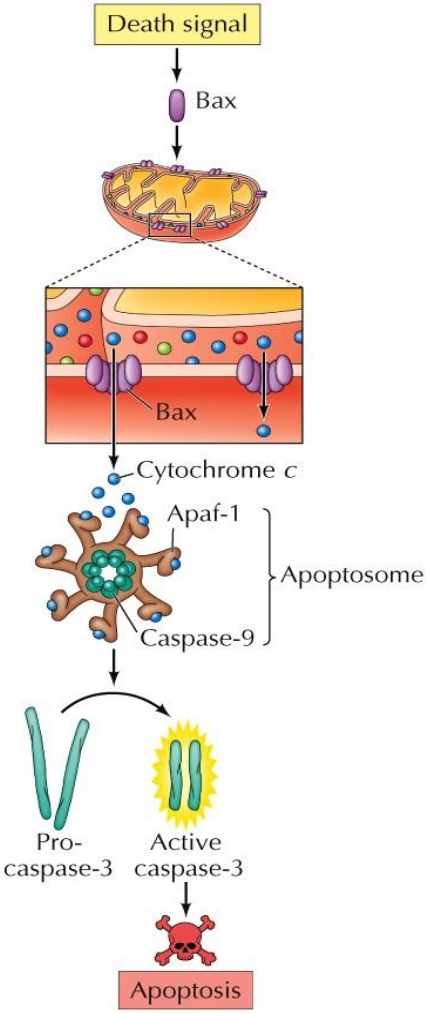
necrosis

Cessation of proliferation (*cytostasis*)



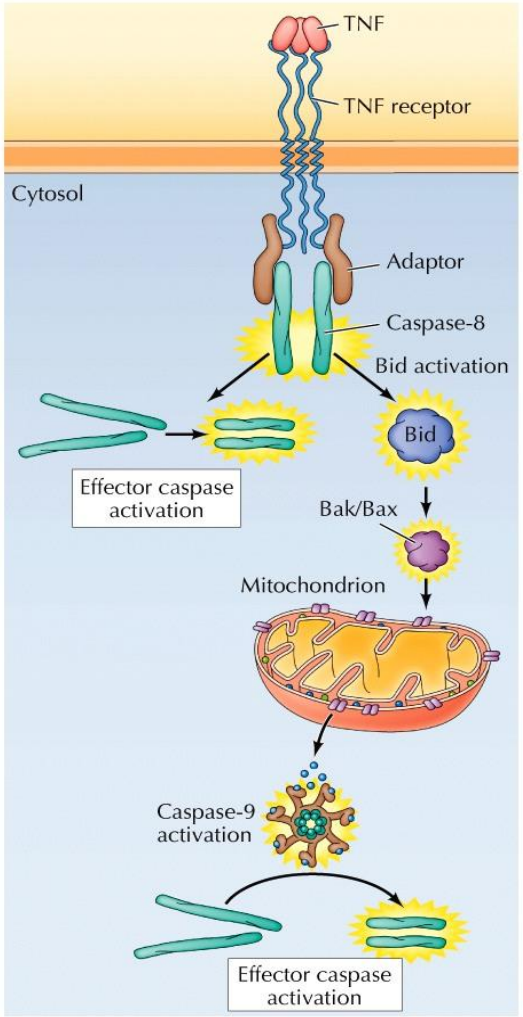
Apoptosis is grouped into two broad **categories**:

Intrinsic



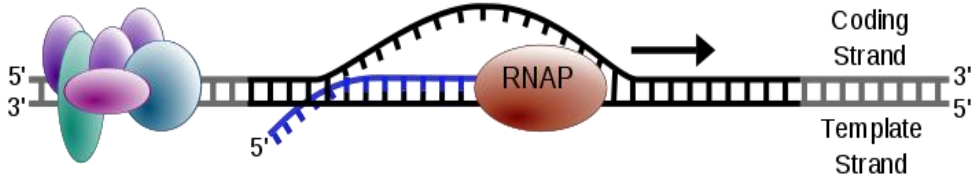
THE CELL: A MOLECULAR APPROACH 6e, Figure 17.7
© 2013 Sinauer Associates, Inc.

Extrinsic

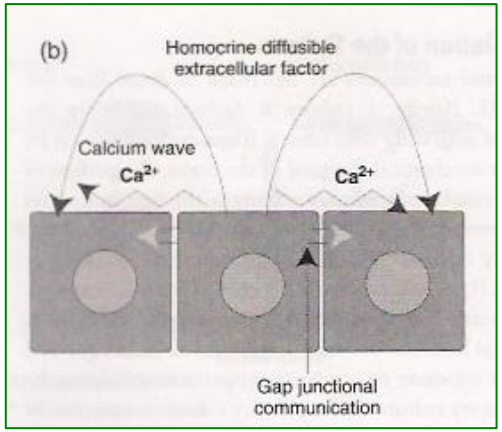


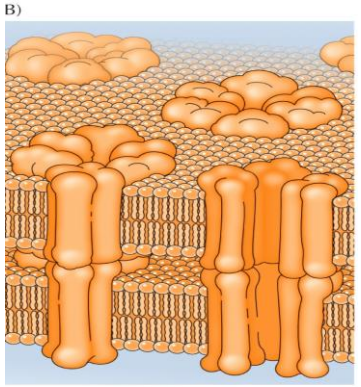
THE CELL: A MOLECULAR APPROACH 6e, Figure 17.11
© 2013 Sinauer Associates, Inc.

Some toxic effects are far more subtle…



- Alterations in gene transcription
- Cell signaling
- Cell-cell interactions
- Genotoxicity

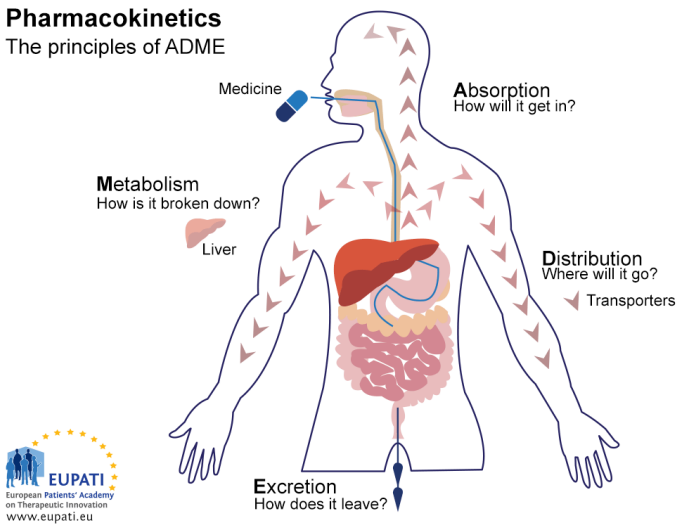


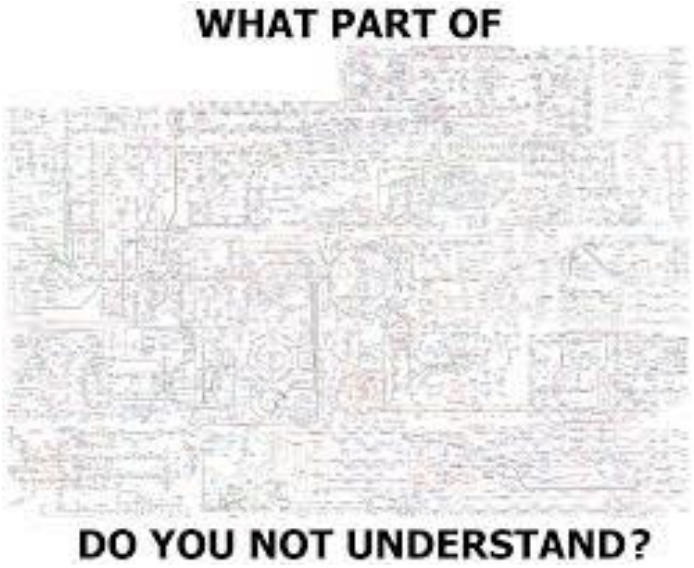


There is a growing understanding in the field that several different assays, designed to measure different forms of cytotoxicity, are necessary.

IN VITRO LIMITATIONS

- **Pharmacokinetics**
- **Metabolism**
- **Tissue Response** (inflammation, fibrosis, organ failure)
- **Systemic Response** (pyrexia, vascular dilatation)





IN VITRO ASSAYS

1. **Viability**
   - An immediate response, such as membrane permeability

2. **Survival**
   - The long-term retention of self renewal capacity

3. **Metabolic**
   - Measures a cellular metabolic response shortly after exposure as an indicator of cell proliferation and/or survival

4. **Genotoxicity and Transformation**
   - Survival in an altered state (e.g. a genetic mutation that leads to alterations in growth control)

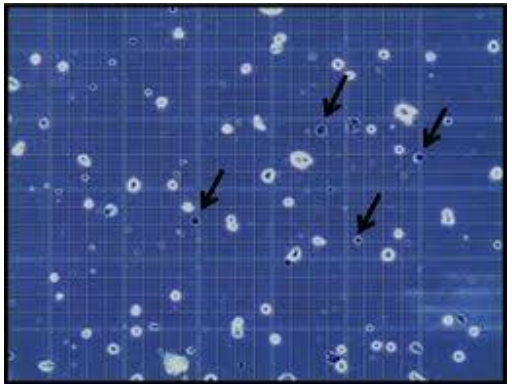
5. **Irritancy**
   - A cellular response analogous to inflammation, allergy or irritation in vivo (Cytokine Release)

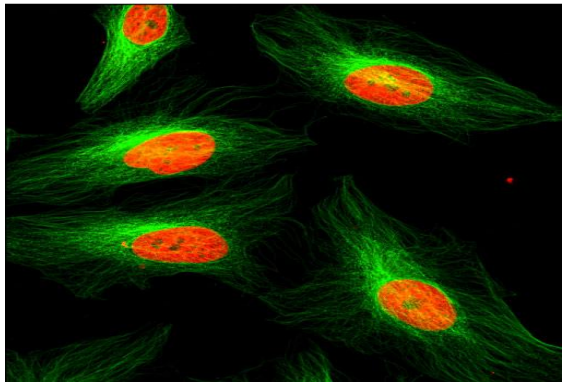
CYTOTOXICITY ASSAYS

viability

Assays which look at the immediate effect of a toxin, generally by measuring the proportion of viable cells.

- Most rely on a breakdown of membrane integrity for dye uptake
- Does not give any information about the long-term cytotoxic response





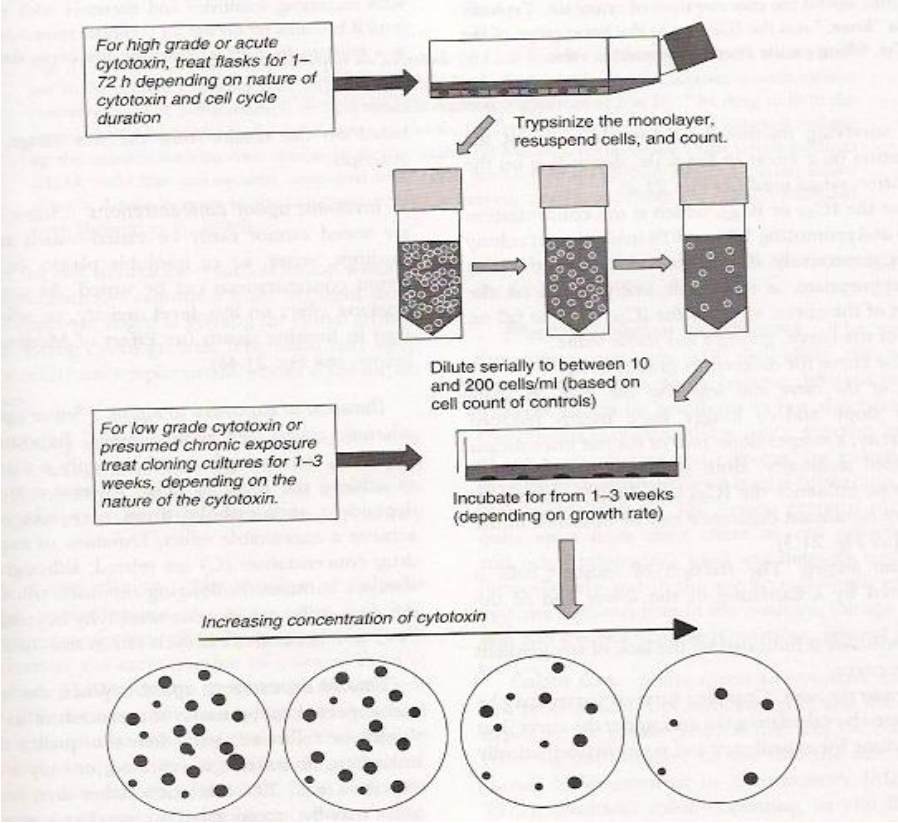
Survival: Retention of an intact, impermeable membrane

CYTOTOXICITY ASSAYS

survival

Assays which look at the long-term effect of a toxin by measuring the retention of regenerative capacity

• Most often measured by determining culture **plating efficiency**



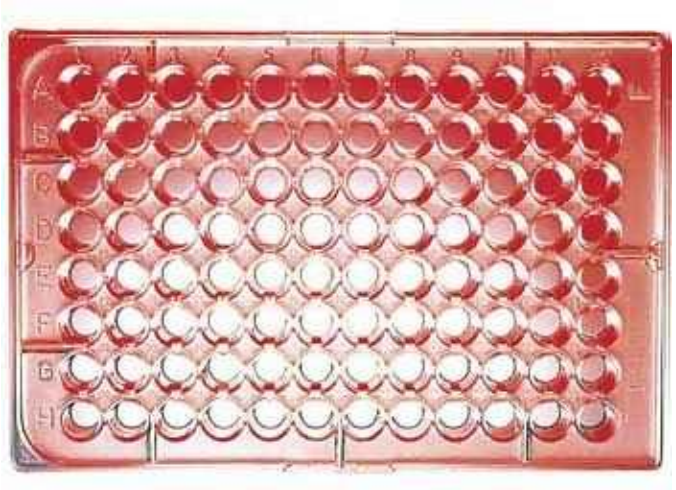
Survival: Retention of regenerative capacity

CYTOTOXICITY ASSAYS

metabolic

Assays which indirectly measure cell survival through metabolic activity

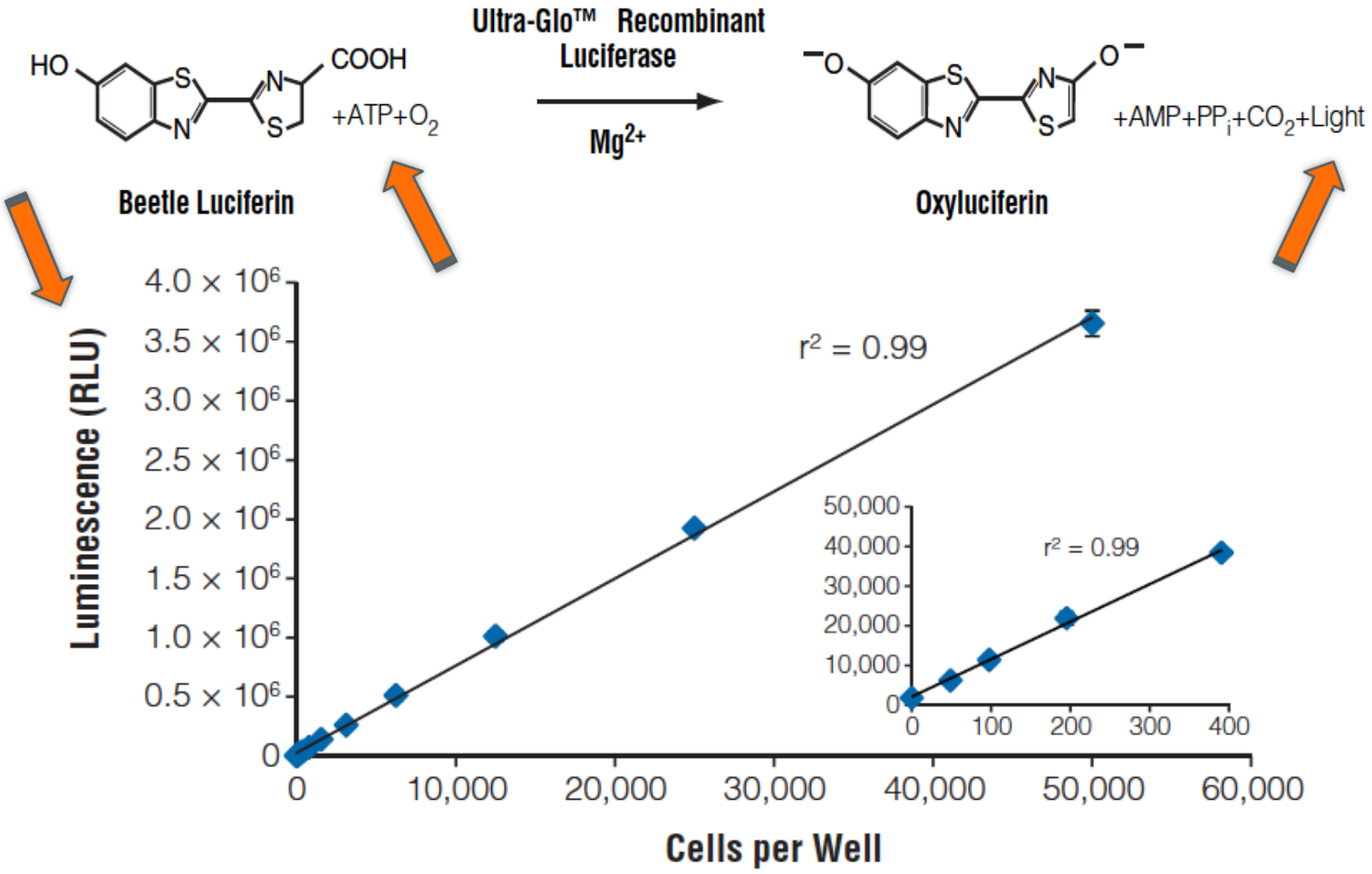
• These assays often involve the use of microtitration plates (96-well, 144-well) which allow for large numbers of replicates and automated handling



Survival: Retention of metabolic or proliferative ability by the cell population as a whole

ATP ASSAY

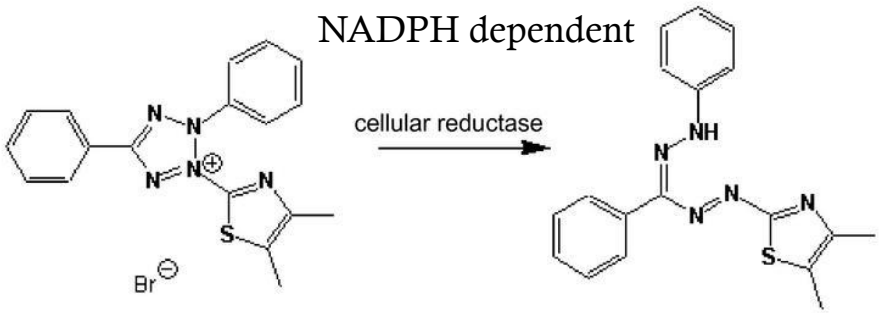
NOTE: Luciferase is an example of bioluminescence and chemiluminescence NOT fluorescence



CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega, inc) 12/12

CYTOTOXICITY ASSAYS

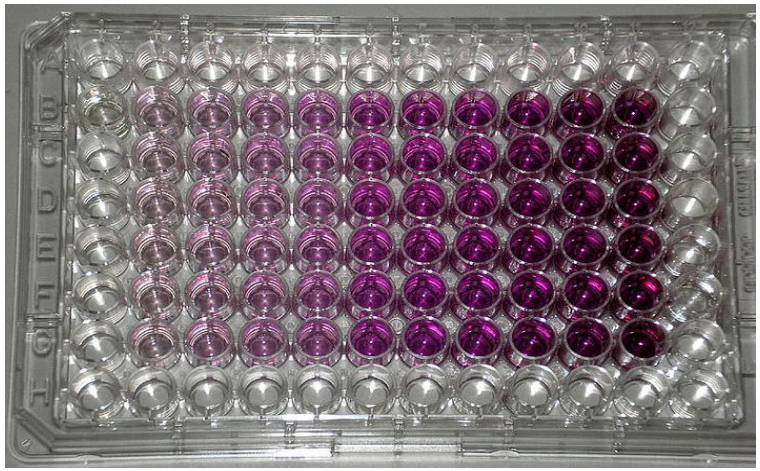
MTT-based



MTT – a yellow, water-soluble tetrazolium dye

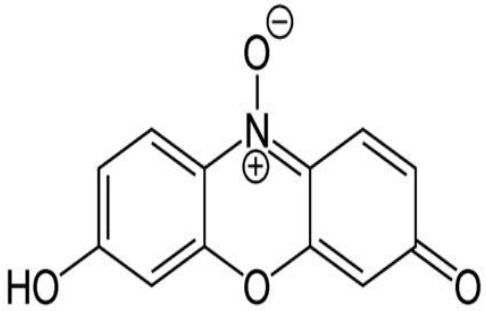
Purple formazan - insoluble

- Colorimetric assay, not fluorescent
- Requires the addition of DMSO… end-point experiment

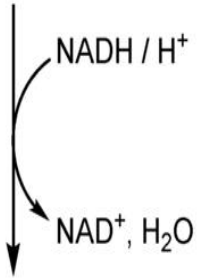


CYTOTOXICITY ASSAYS

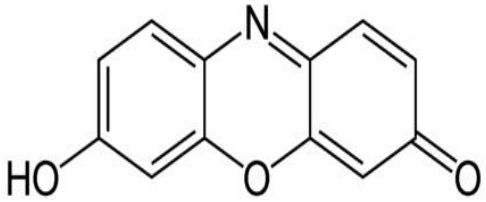
Alamar Blue



Resazurin



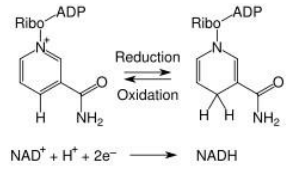
(REDOX)



Resorufin

- Nontoxic to living cells and allows for longer studies
- Both a colorimetric assay and fluorescent assay

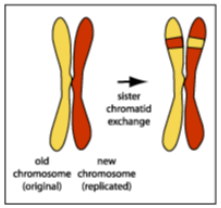




NAD+/NADH Reduction/Oxidation Reaction

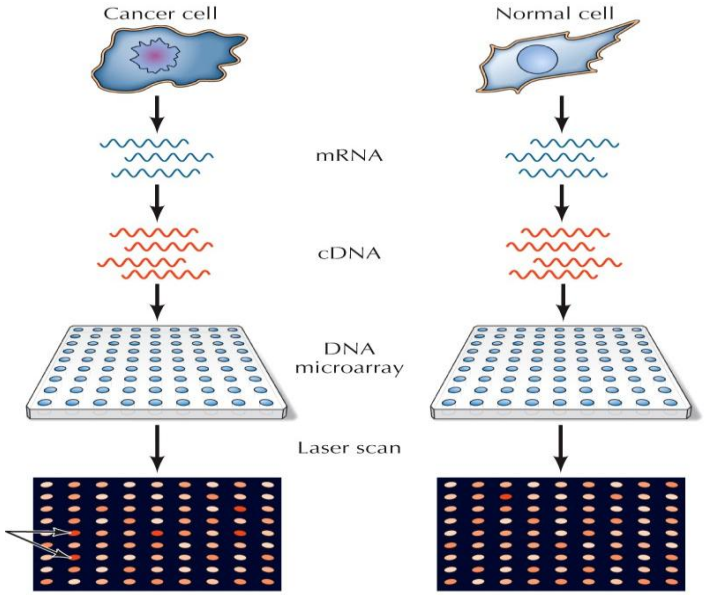
CYTOTOXICITY ASSAYS

genotoxicity



Assays which assess the DNA damage due to a toxin.

• Damage can be measured based on p53 upregulation, in vitro demonstrations of transformation (anchorage independence, reduced density limitations), or mutagenesis (sister chromatid exchange assay)



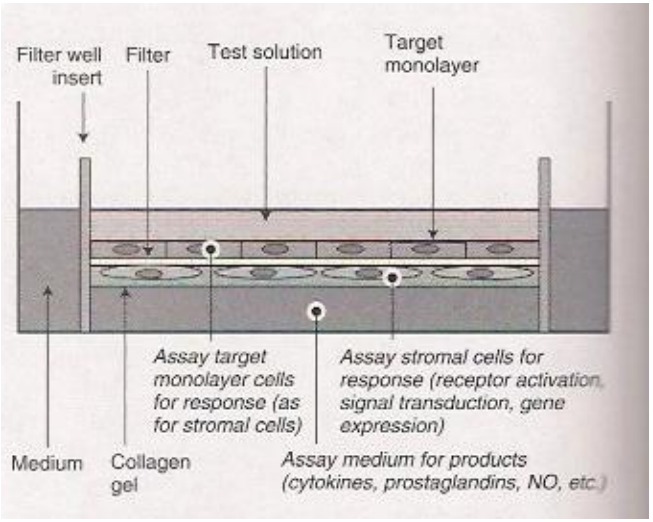
THE CELL: A MOLECULAR APPROACH 6e, Figure 4.26
© 2013 Sinauer Associates, Inc.

Survival: **Retention of normal genotype and phenotype**

CYTOTOXICITY ASSAYS
inflammation

There is a need for tissue culture testing to reveal inflammatory responses induced by pharmaceuticals, cosmetics, etc. Currently, in vivo models are the only reliable measure of inflammation.

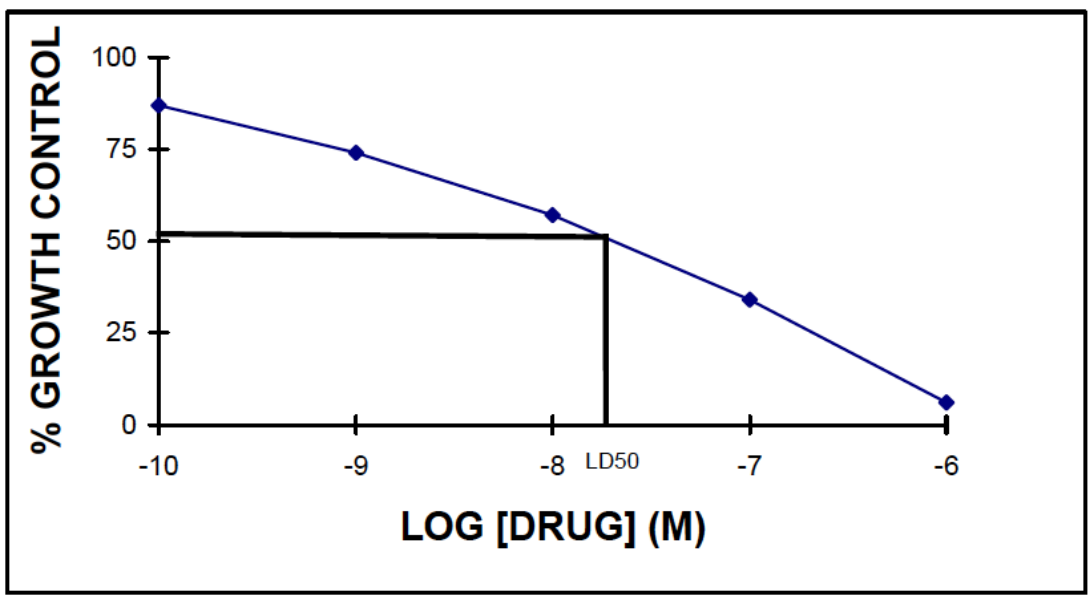
• Organotypic assays are being developed to measure a cytokine response



Survival: **Minimal inflammatory response**

Pointers for today

- Make your TX100 dilutions directly into the wells of your plate.
- We do not have a multichannel pipet, you may want to develop a team pipetting system. Who in your group is a good robot?
- When you get to lab start to set up of your Alamar Experiment.
  - After you have your plates set up, we will take part of the class to demonstrate the use of the plate reader.
  - The other half of the class can take pictures of their proliferation flasks.

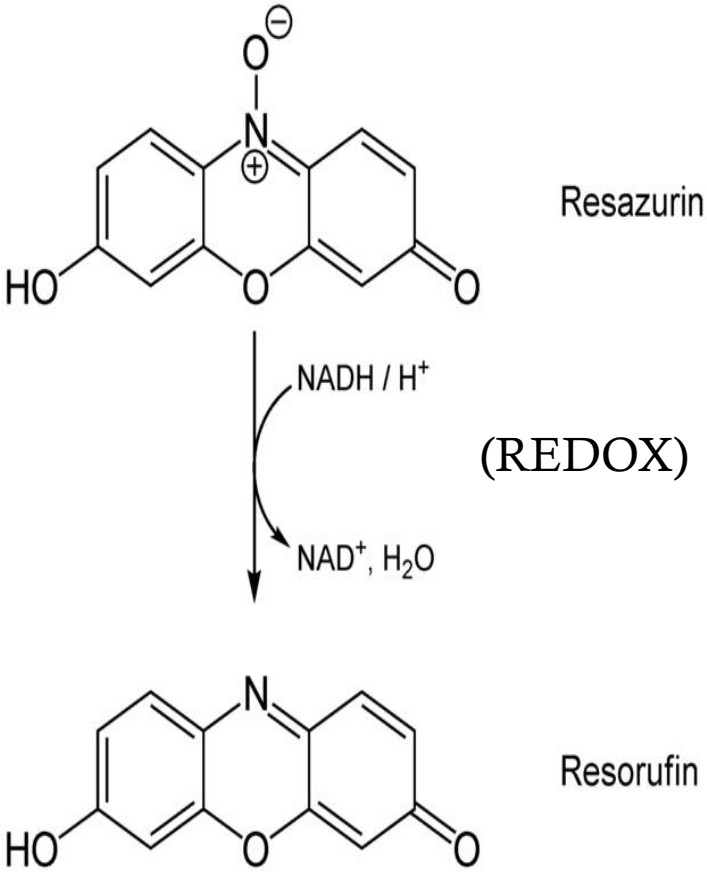


Survival Curves

Alamar Blue Data

CYTOTOXICITY ASSAYS

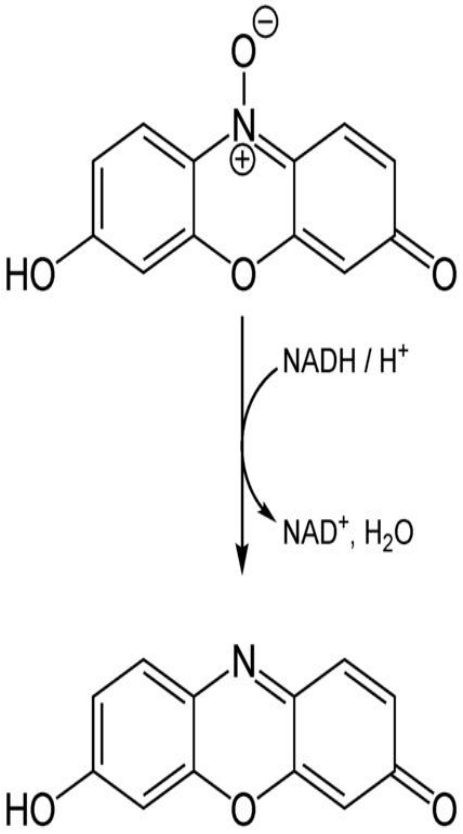
Alamar Blue



- Nontoxic to living cells and allows for longer studies
- Both a colorimetric assay and fluorescent assay



Alamar Blue:Redox

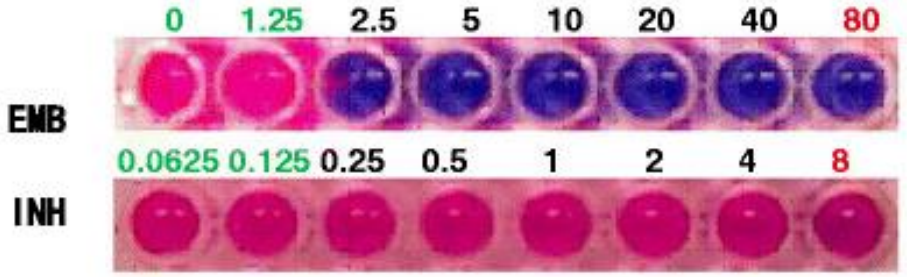


Resazurin

Resorufin

Reduction: Gain of electrons
Oxidation : Loss of electrons

Resazurin: Blue and weakly fluorescent
Resofurin: Pink and fluorescent at 590nm



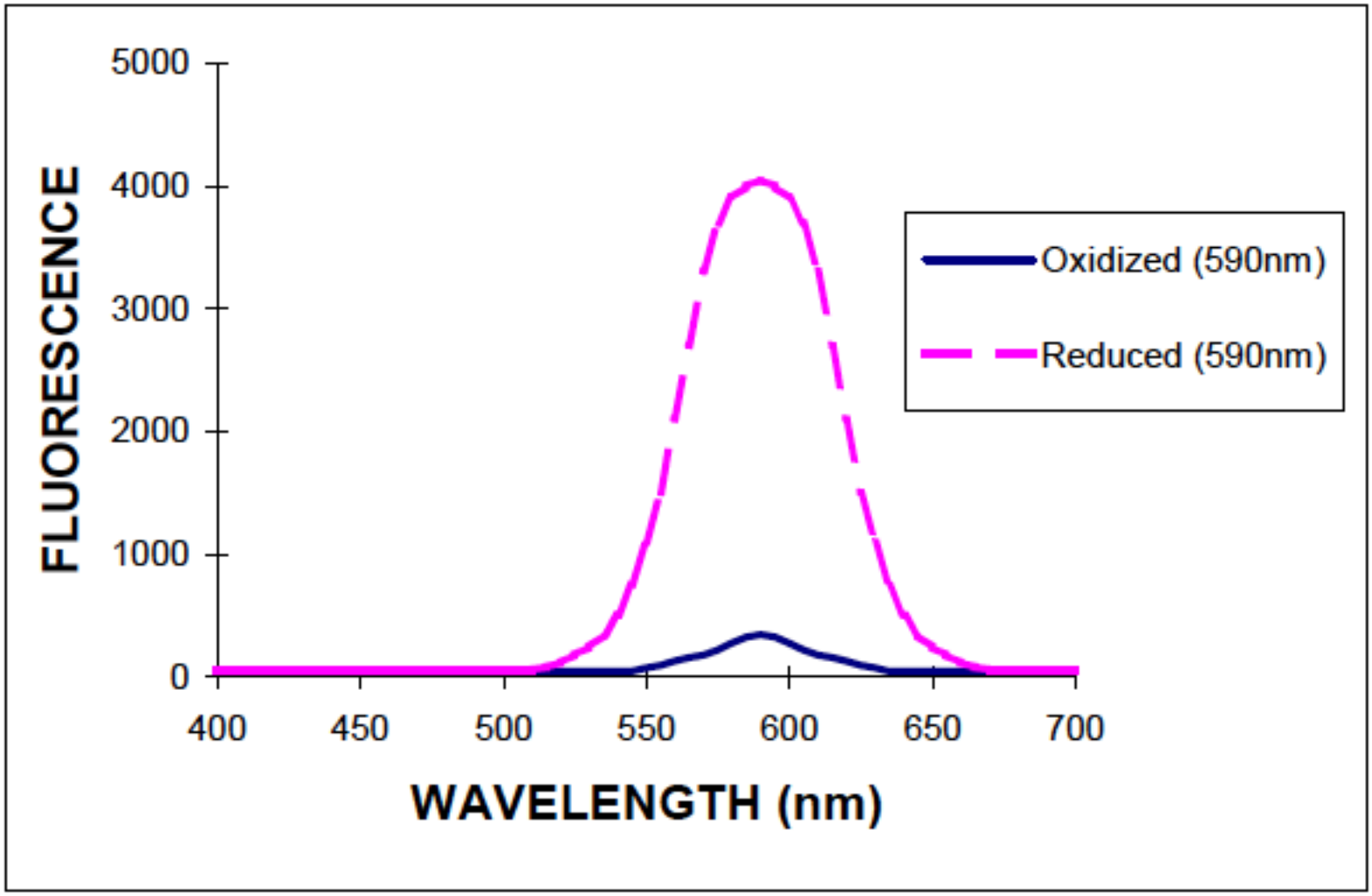
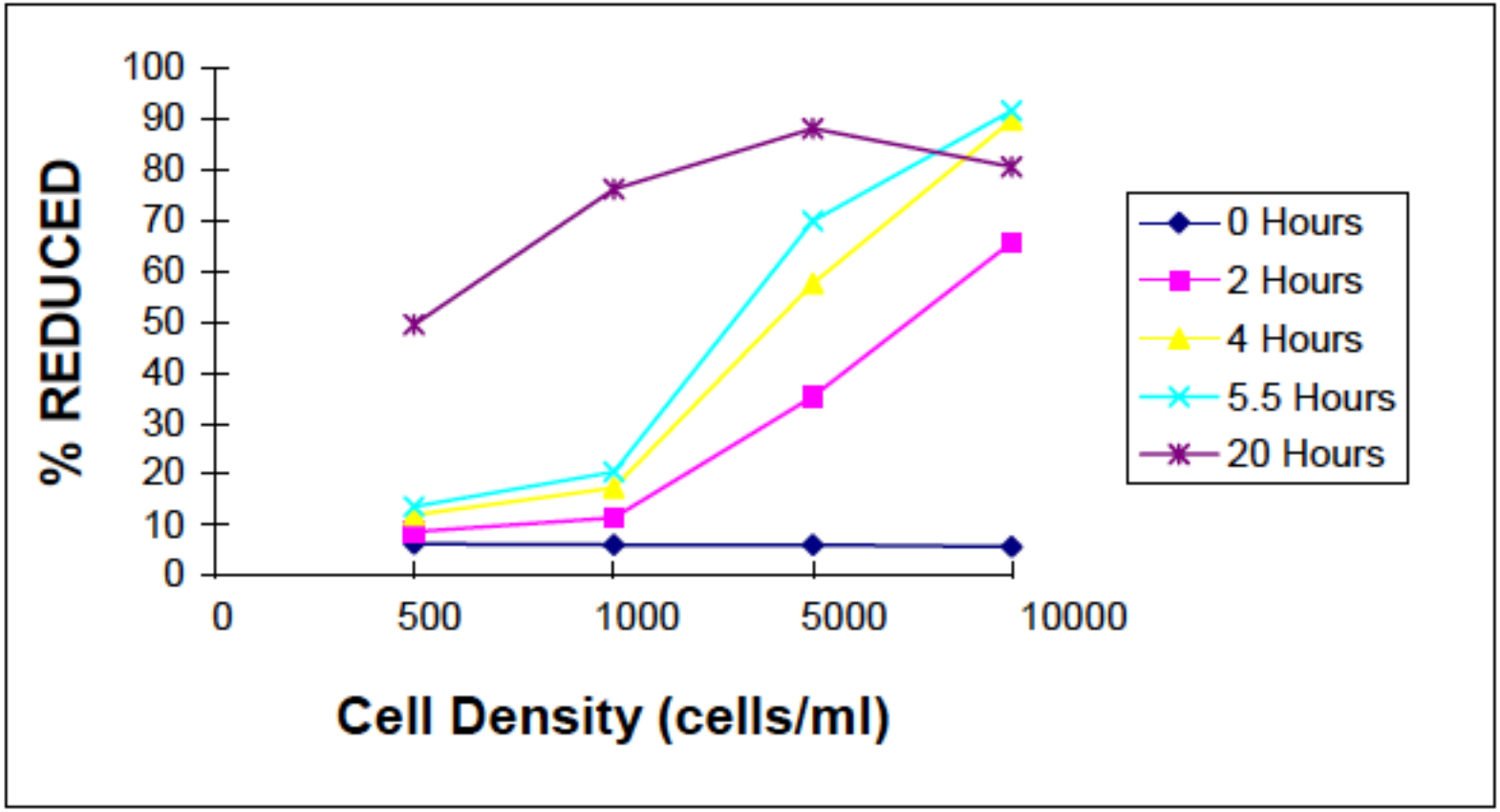
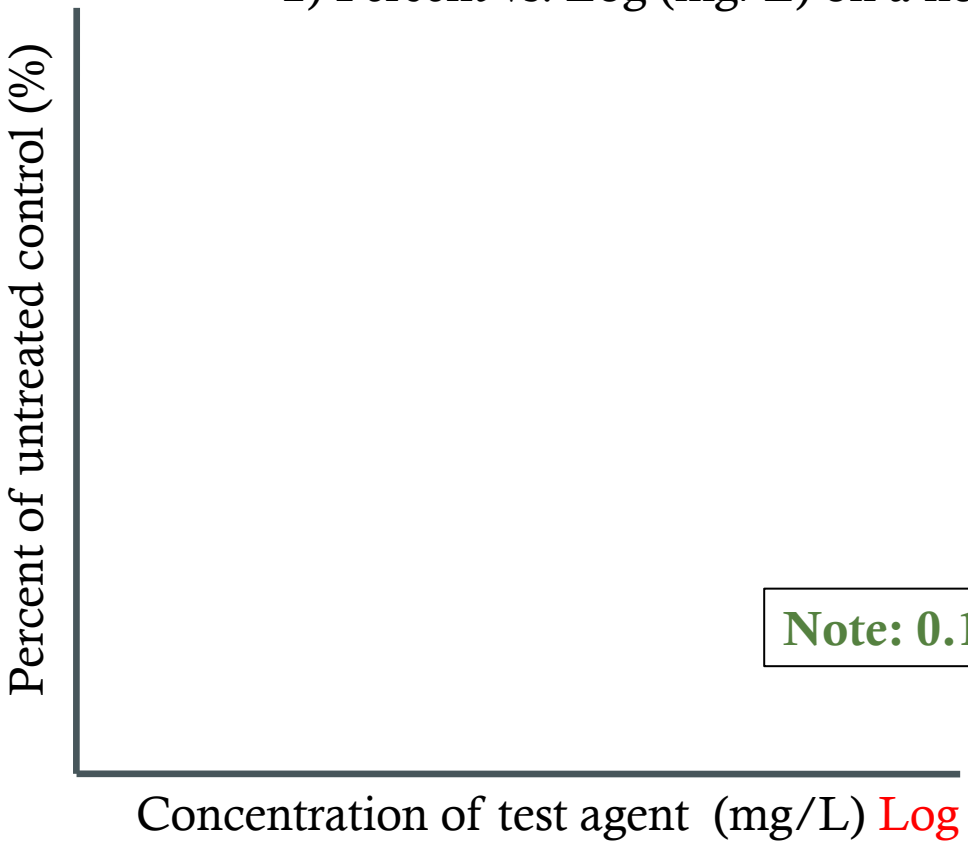


Figure 1a. alamarBlue® Fluorescence Emission Spectra


% REDUCED
100
90
80
70
60
50
40
30
20
10
0
0
500
1000
5000
10000
Cell Density (cells/ml)
0 Hours
2 Hours
4 Hours
5.5 Hours
20 Hours

Our Alamar Data

Generate two graphs: 1) Percent vs. mg/L TX on a log scale
2) Percent vs. Log (mg/L) on a normal scale



Note: 0.1% (w/v) = 1mg/ml

Our Alamar Data

Calculate percentage of untreated (negative) control with the following formula:

$$\frac{\text{(Avg Fl 590 of test agent dilution-Avg FL590 of no cell control)}}{\text{(Avg Fl 590 of untreated control-Avg FL590 of no cell control)}} \times 100$$

- We are taking the percentage of "true" Alamar fluorescence in treated vs. untreated cells (columns 1,2).
- Autofluroescence is removed by takin the average fluorescent value from the no cell control (column 12)
- Your data will come to you in an excel file. I suggest that you use the excel file to organize your calculations.
- The final percentage will be graphed on the y-axis of your alamar graph.

Alamar Graph Steps

- Calculate your percentage of the negative control with the formula (Y-axis)

- The final concentration of TX100 in mg/L is listed on your experiment sheet for each text column.
  - Generate another graph with your calculated percentages of fluorescence against the Log of the concentration of TX100 on a normal scale.

LD50, IC50, EC50?

Determine the LD50 endpoint from the graph by reading from where the 50 percent point intercepts the Dose Response Curve to the concentration along the x-axis. That concentration is the LD50 value. (Figure 6)

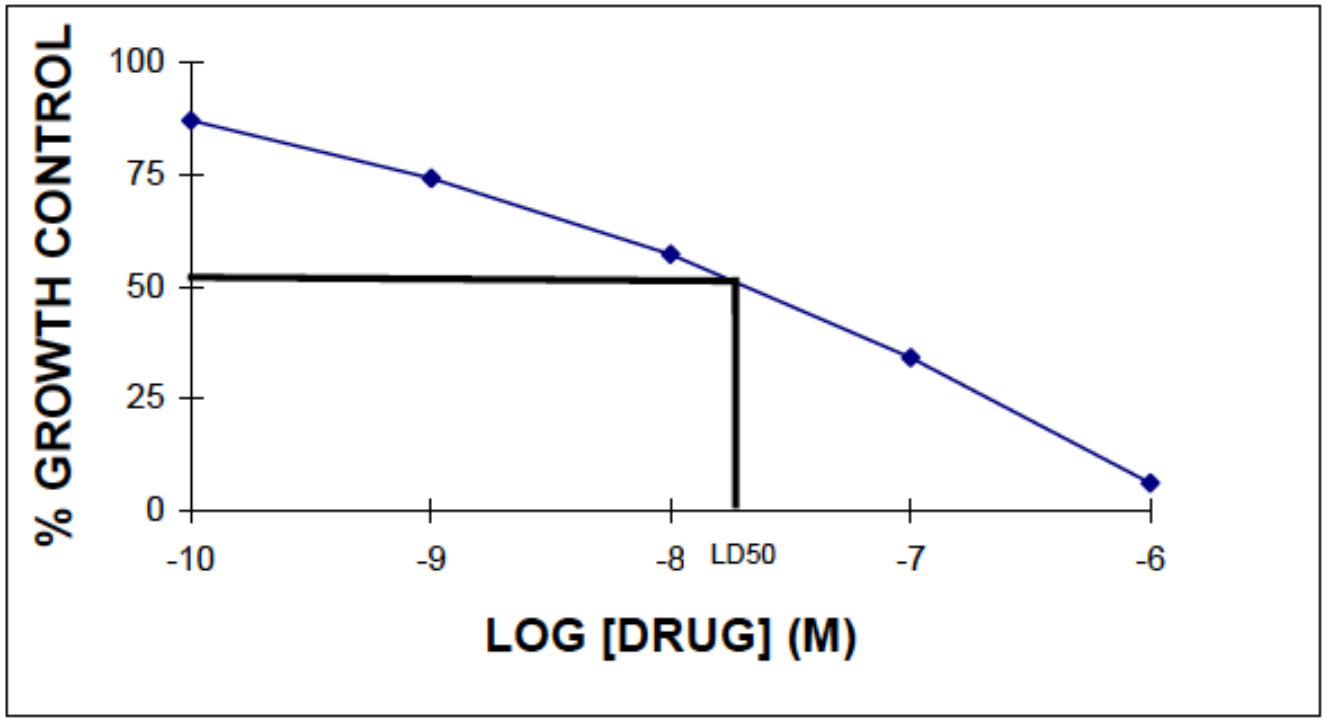
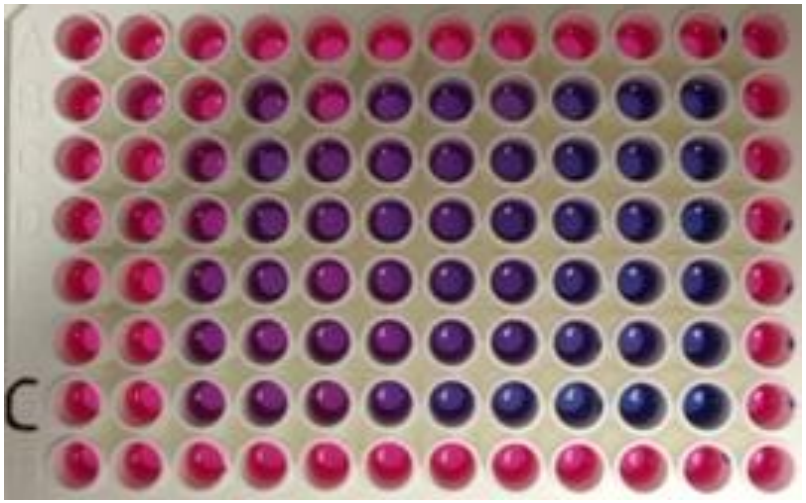
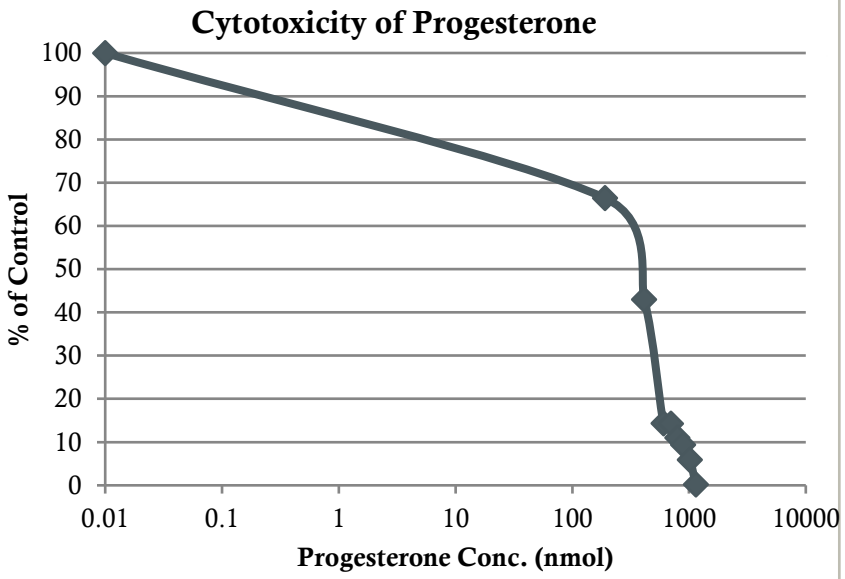


Figure 6. Determination of Doxorubicin LD50 Using alamarBlue®

How to Present Your Data





Conc (nmol)	0.01	190.8	413.4	604.2	699.6	795.1	890.4	1017.6	1144.82	1208.4	1399.2	0.01
4 Hours	1	2	3	4	5	6	7	8	9	10	11	12
A	35899	40513	43230	42916	47950	43378	42265	42732	42056	43986	36401	36048
B	42267	15525	35257	14819	19540	10888	12132	9666	9103	10833	10193	39931
C	49932	45826	17847	11827	10868	11276	11413	8740	7718	9379	9357	34082
D	45717	18182	22973	9950	12780	11012	10695	8153	8191	9003	5878	36958
E	43152	37044	16839	13145	14722	12159	10553	12631	7759	8556	8627	33440
F	51139	31530	17338	14272	12775	13347	11163	11181	8368	7914	6815	33395
G DMSO	43261	27572	17965	11353	11899	8985	7649	7240	6239	6244	4873	36157
H	47139	43090	39907	37694	42085	39013	43303	37515	37895	32981	36318	32784

The graph (top right) is all that is needed. If there is any confusion in the data, the raw table (left) will be helpful so save it.

Stats?

In this experiment we have 8 repeats of each concentration. Using these repeats, you may be able to generate some statistical information.

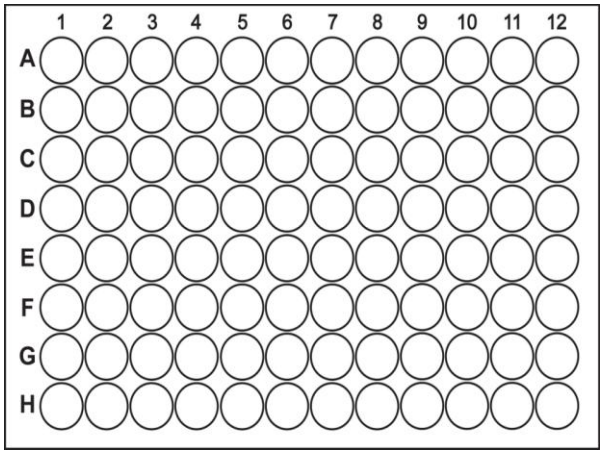
For this results section, generate error bars.

What about statistics??

First.. how can we determine the "center" of our values?

mean…median…mode

A1 2140
B1 1906
C1 2207
D1 1659
E1 1849
F1 2010
G1 2049



mean = _____ median = _____ mode = _____

What about statistics??

Second.. establish the variance within you data

• Importantly, ask yourself if the data points are independently repeated experiments or are they replicates.

→ n=1 OR n=7



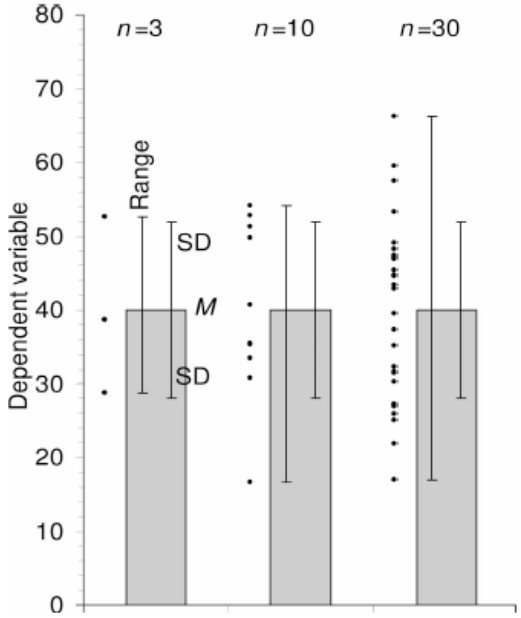


What about statistics??
error bars

Descriptive Error Bars

- give information describing the data
- most commonly use range or standard deviation (SD)

$$SD = \sqrt{\frac{\sum (X - M)^2}{n - 1}}$$



Descriptive error bars do not help you to compare groups

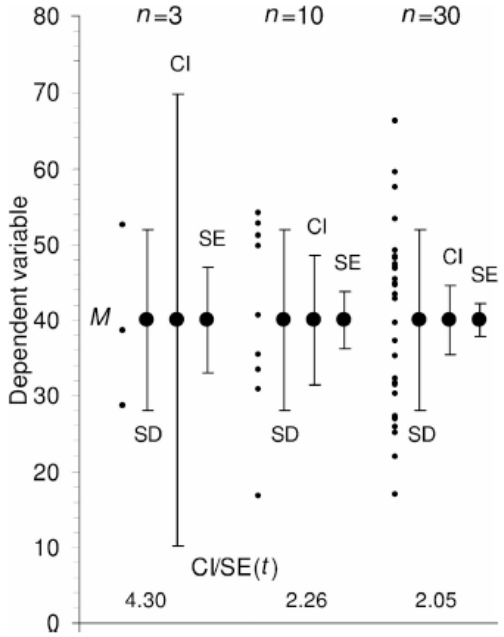
What about statistics??
error bars

Inferential Error Bars

- gives information about what conclusions, or inferences, are justified
- most commonly use standard error of the mean (SE) or 95% confidence intervals (CI)

$SE = SD/\sqrt{n}$

$CI = M \pm t_{(n-1)} \times SE$



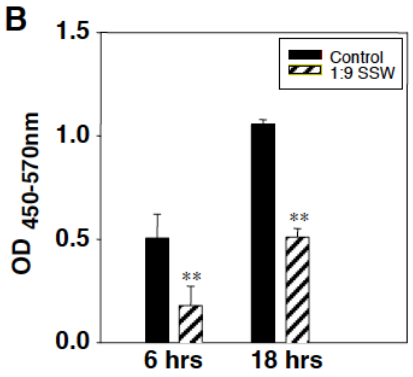
SE is the most commonly used error bar for comparing groups

What about statistics??
P-values

The probability that you will get, by chance, a difference as large as the one you observed between two groups.

By convention, $P<0.05$ you say the result is statistically significant. If $P<0.01$ you say the result is highly significant.

I wont stop you, but.. let's save this for another time



What about statistics??

Rule #1:
When showing error bars, always explain in the figure legend what they are (SD? SE? CI?).

Rule #2:
The value of *n* (sample size) must be stated in the figure legend.

Rule #3:
Because experimental biologists are usually trying to compare results with controls, it is generally appropriate to show inferential error bars (SE, CI) rather than descriptive error bars (SD, range).