Tips for Multiplexing Cell-Based Assays:
Plan for success

Fall 2010

Promega

Click this icon to view speakers notes for each slide.

Considerations for Successful Cell-Based Assays

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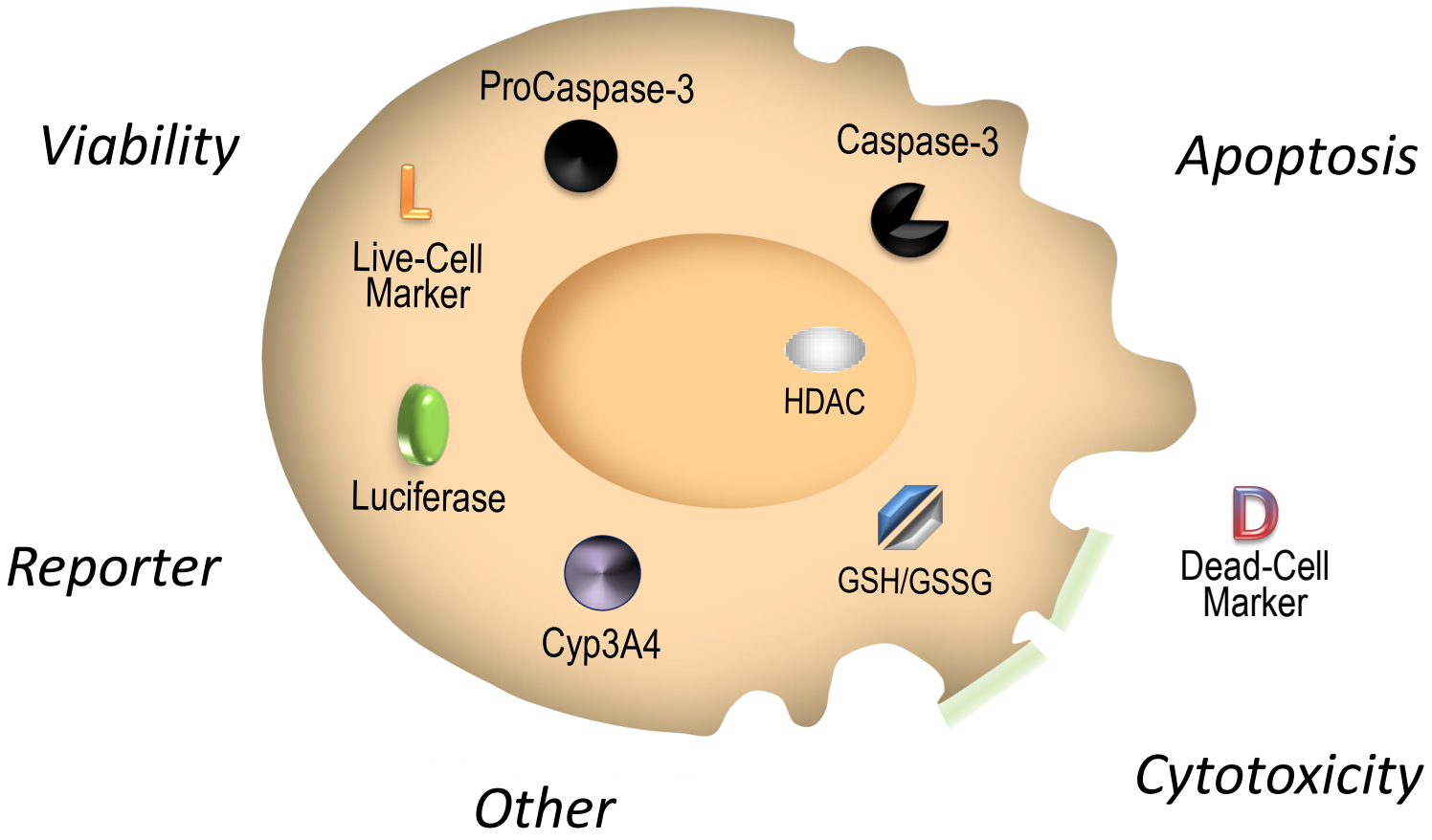
Assay Choices



Considerations for Successful Cell-Based Assays

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Assay Design/Process

Type of Cell

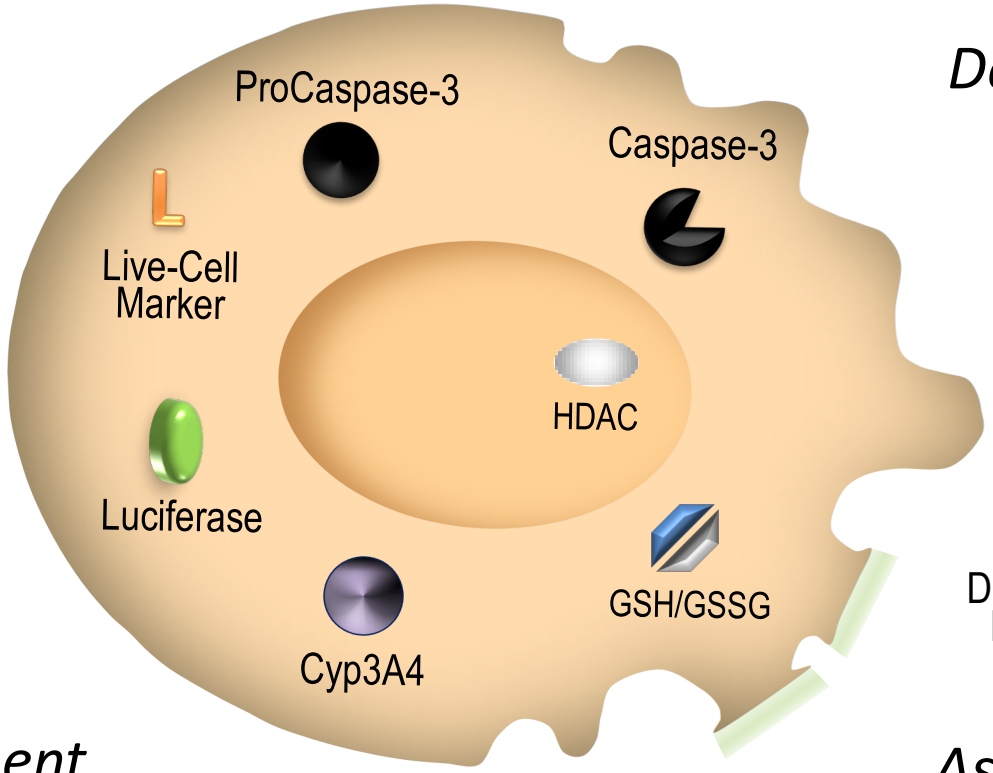
Culture Variables

Treatment Parameters

Detection Parameters

Controls

Assay Technique



Considerations for Successful Cell-Based Assays

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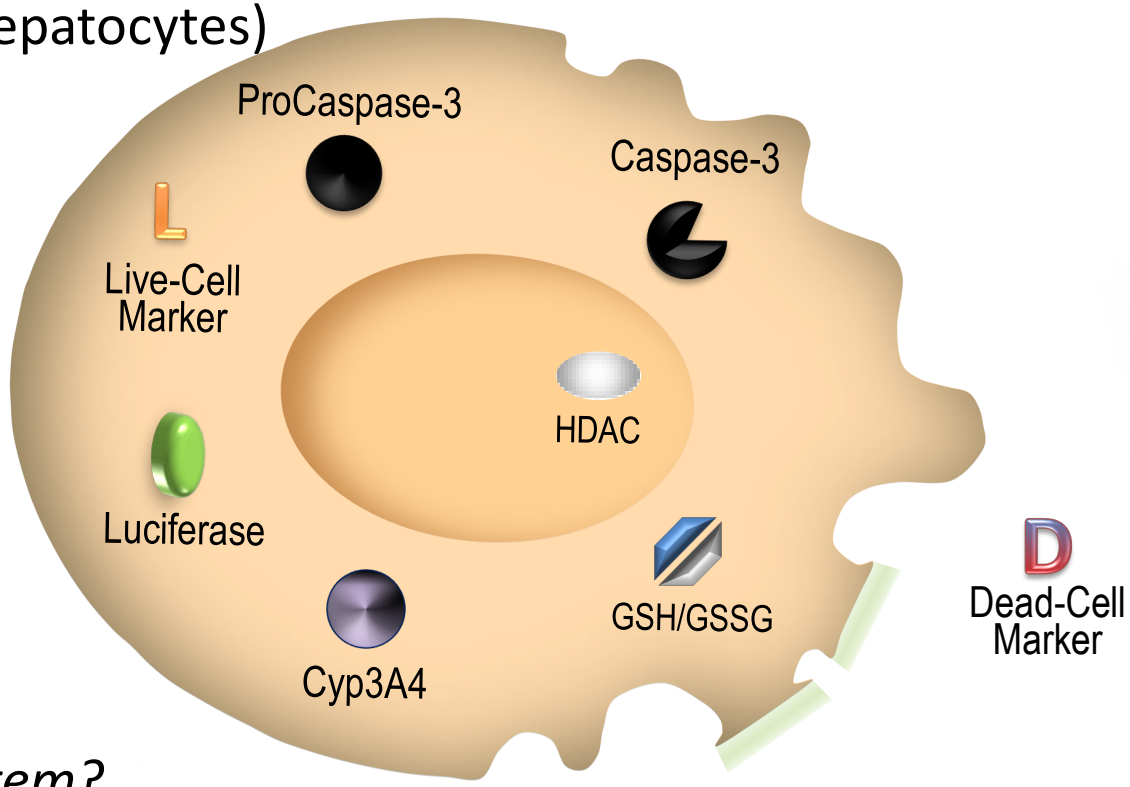
- Fibroblasts (HEK293, Cos)
- Cancer cell lines (HepG2, PC-3)
- Primary cells (HUVECs, hepatocytes)

Type of Cell



- *Amenable to assay?*
- *Faithfully represent system?*
- *Express factors, signaling intermediates?*

Choice of cell type influences assay design

- **Viability** - metabolism higher in cell lines, lower in primary cells
- **Cytotoxicity** – background higher in primary cells
- **Apoptosis** - differences in susceptibility to inducers

Assay parameters that may need to be altered:

- Seeding density
- Treatment choice, concentration, duration
- Assay incubation time

Considerations for Successful Cell-Based Assays

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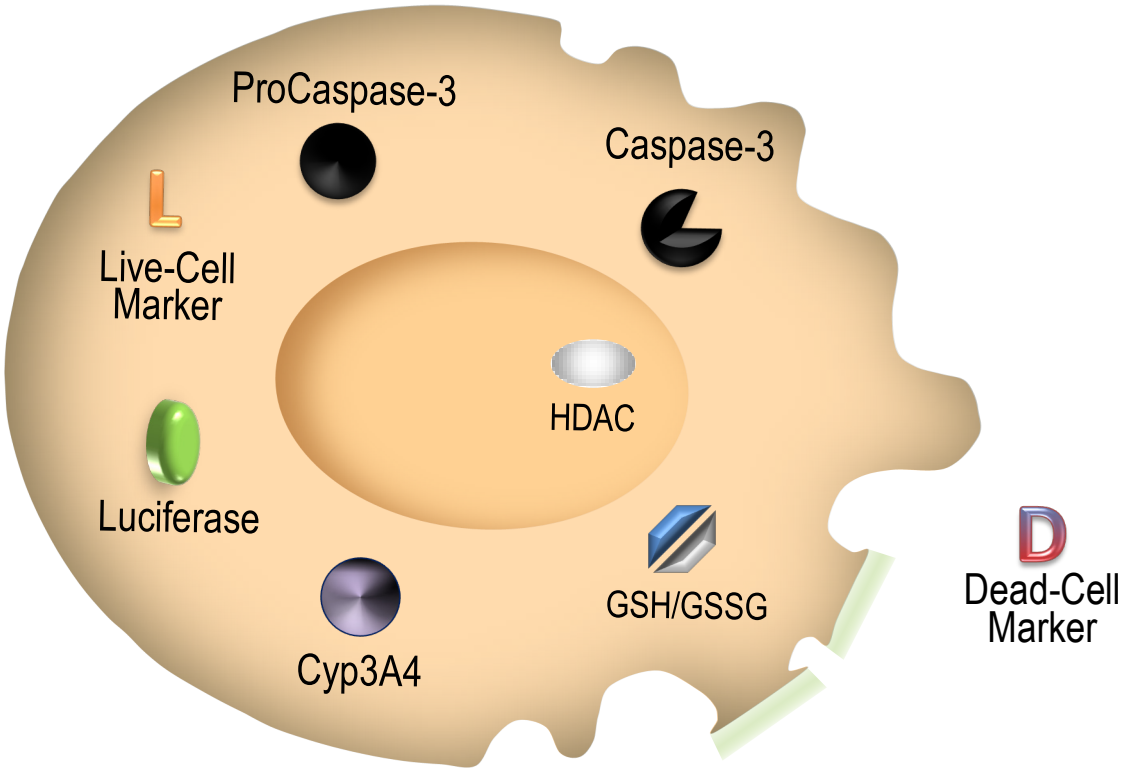
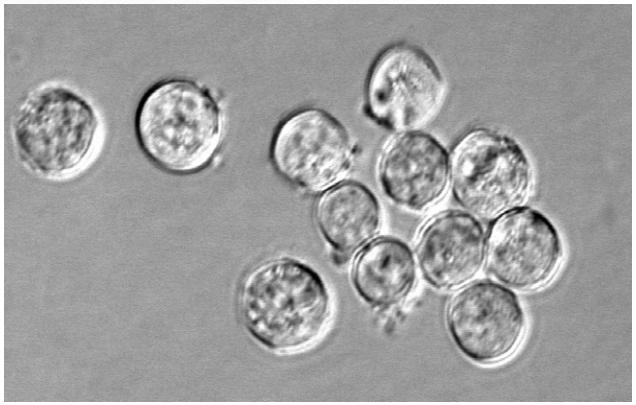
Culture Variables



Basic Cell Culture Considerations

- Cell health
  - Medium
    - *High quality, fresh*
    - *Consistent source*
  - Handling
    - *Trypsinization*
    - *Pipeting*
    - *Counting (viable cells)*
  - Contamination
- Cell line identity
  - *Verification required by NIH, many journals*



Consistent, careful handling of the cells is crucial for reliable, repeatable results

More Cell Culture Considerations

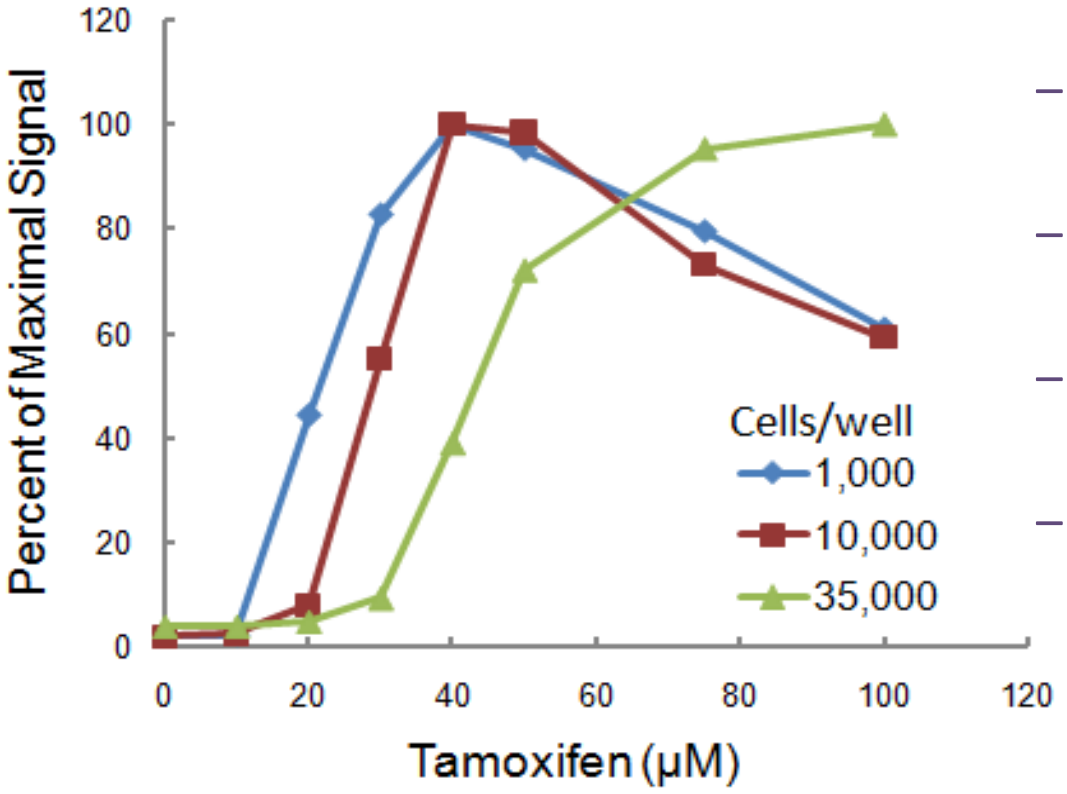
- Cell line passage
  - As passage number increases, cells may change character:
    - *Change in response to treatments?*
  - *Line may change character if allowed to reach confluence*

    - *Use low passage number – keep passage number consistent*
    - *Keep track of cultures when bulking up & during experiment*

- Cell density
  - Seeding density & cell density at time of treatment
  - Will the cells become confluent by treatment?
    - *Will cell density influence response to treatment?*
    - *Will the culture be transfected? (% confluence important parameter)*
    - *Do cells need to be equilibrated, or assay begun immediately?*

Cell number/density may alter dose response

- Induction of apoptosis as measured by Caspase-Glo® 3/7 Assay
- Replicate Dose Response Curves (DRC) from different seeding densities



- *Sparser cultures show increased sensitivity*
- *Higher densities may provide protective effect…*
- *Higher density cultures may need longer exposure*
- *Overgrown cultures will have a higher background*

Cell number optimization for viability and/or cytotoxicity assay

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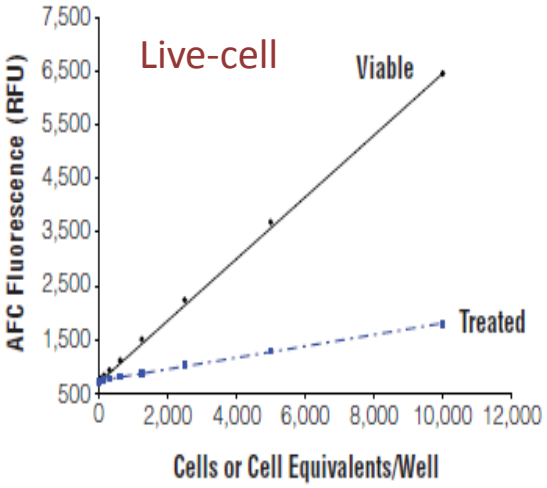
Control assay with serial dilution of cells

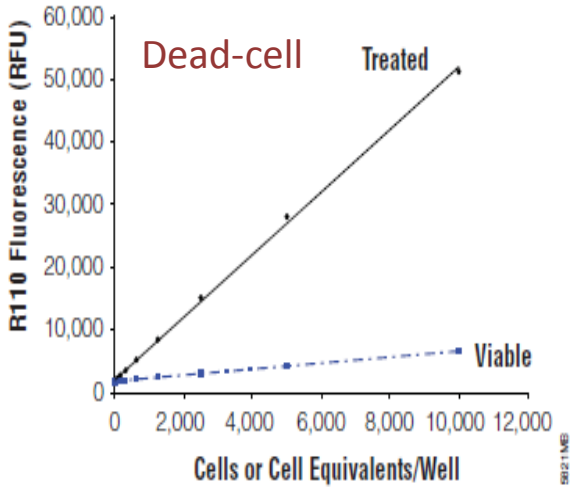
- *Determines sensitivity & range of assay*
- *Guides decision on seeding density, timing between plating, treatment, assay*

For live/dead multiplex, duplicate sets, one treated & one untreated

Table 1. Schematic of 96-well plate layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A	10,000 Cells/Well											
B	5,000 Cells/Well											
C	2,500 Cells/Well											
D	1,250 Cells/Well											
E	625 Cells/Well											
F	313 Cells/Well											
G	156 Cells/Well											
H	0 Cells/Well											
	untreated						treated					





Considerations for Successful Cell-Based Assays

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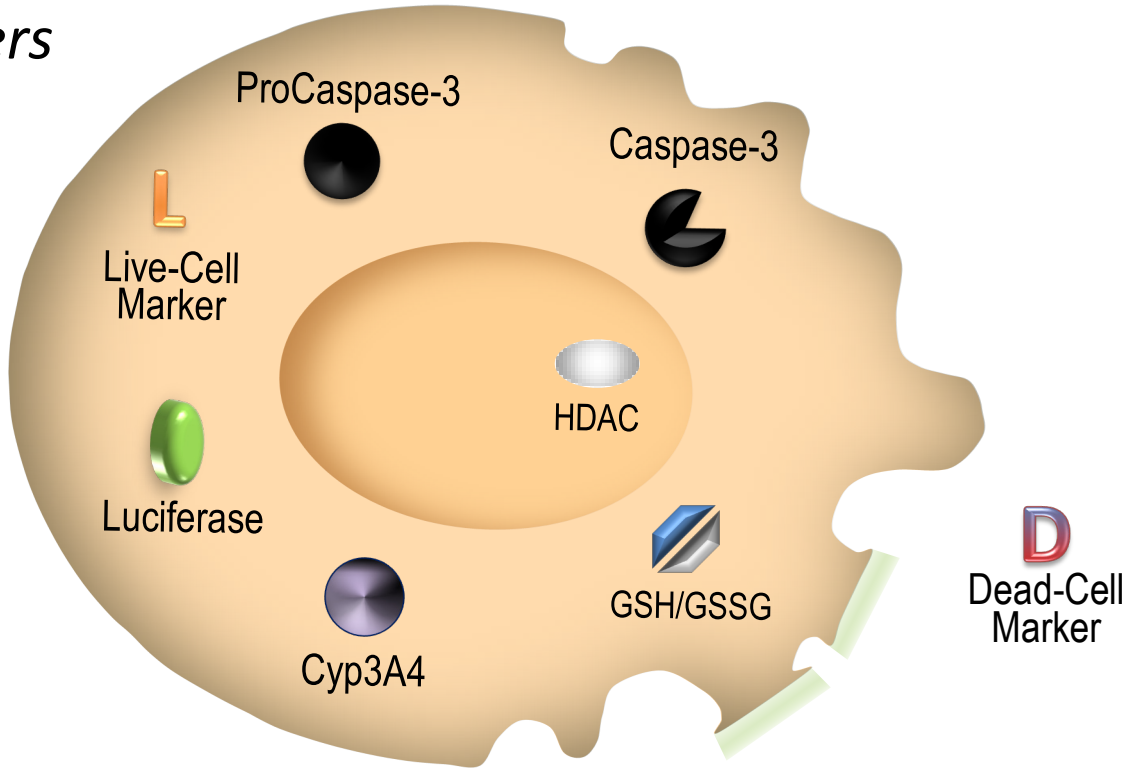
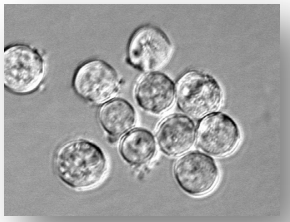
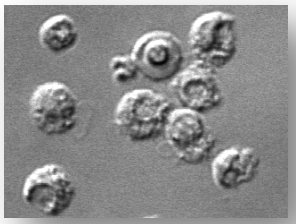
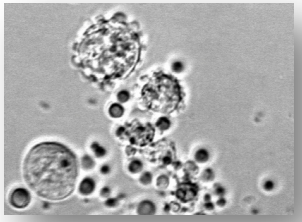
Treatment Parameters



Treatment Parameters







Dose

- Affects the synchrony & timing of response
- May even determine *type* of response
  - *Some drugs which induce apoptosis may be acutely cytotoxic at higher doses*

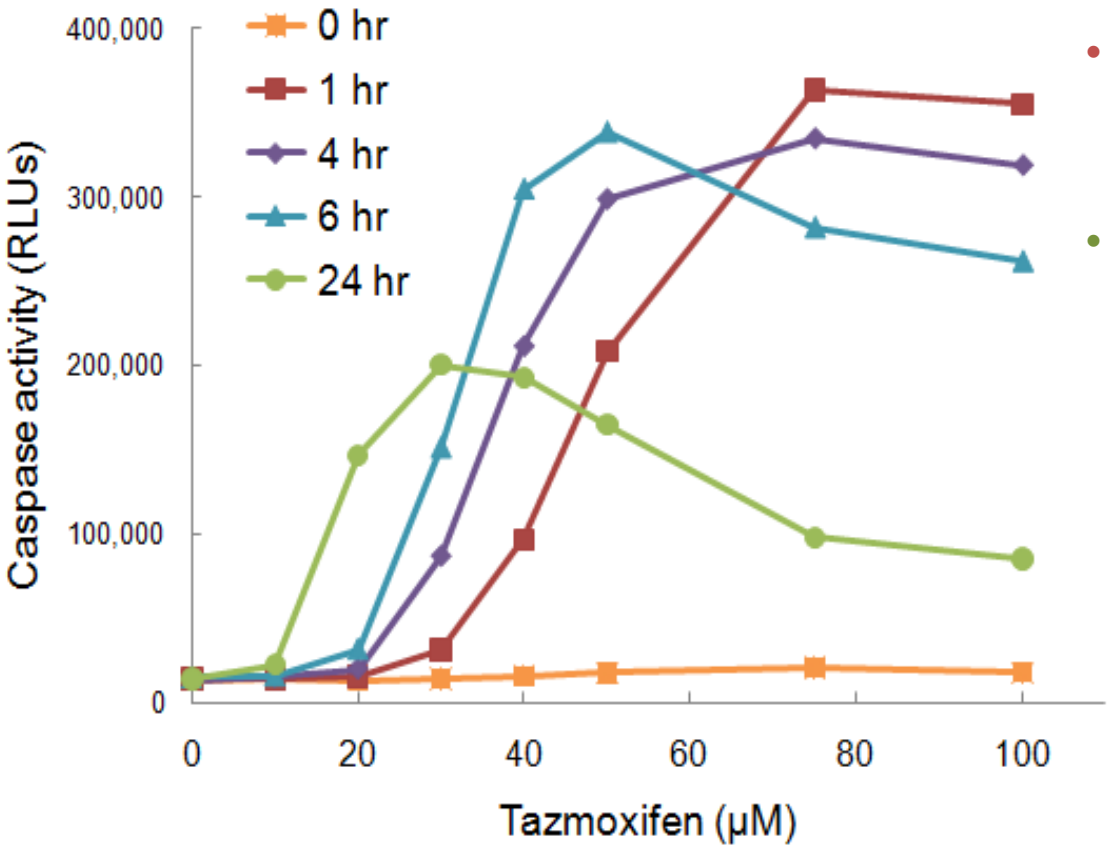
Timing

- Treatment → Assay
  - *Optimal timing affected by type of cell, compound, dose*

All assay parameters may interact, but dose timing & cell type are the most important & intertwined

Timing and dose interact to shape response

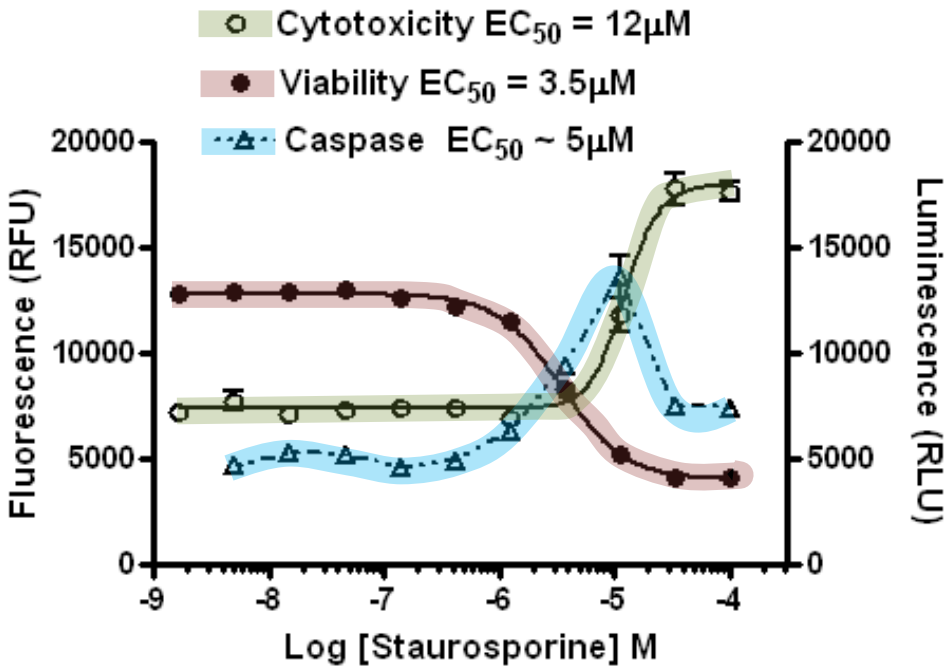
- Induction of apoptosis as measured by Caspase-Glo® 3/7 Assay
- Replicate Dose Response Curves (DRC) measured at different times



- *At 1 hour max response at higher doses; no response seen at lower doses*
- *At 24hr max response at lower doses; at higher doses response missed, analyte has greatly diminished*

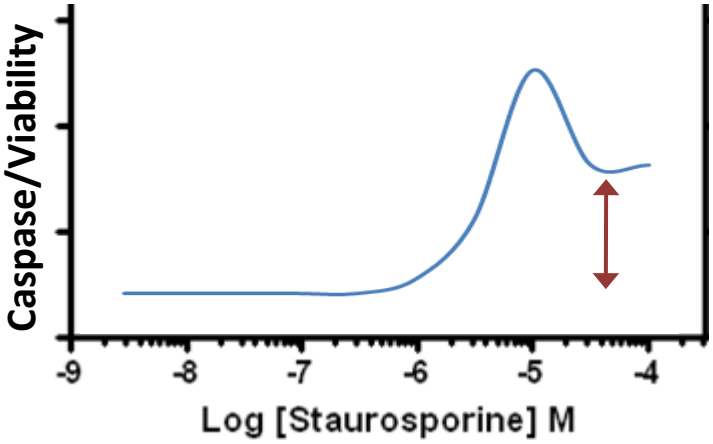
DRC & timecourse control assays are often worth the investment

Multiplexing makes more robust assays: Caspase data compared to live/dead data



- *Induction of apoptosis is asynchronous in a population of cells*
- *Cells undergoing apoptosis are still "alive"*

- *At highest doses caspase signal is low …*
- *But it is because most cells have already completed 2° necrosis…*
- *Normalizing caspase to viability score shows there is still a strong induction at those doses!*



Considerations for Successful Cell-Based Assays

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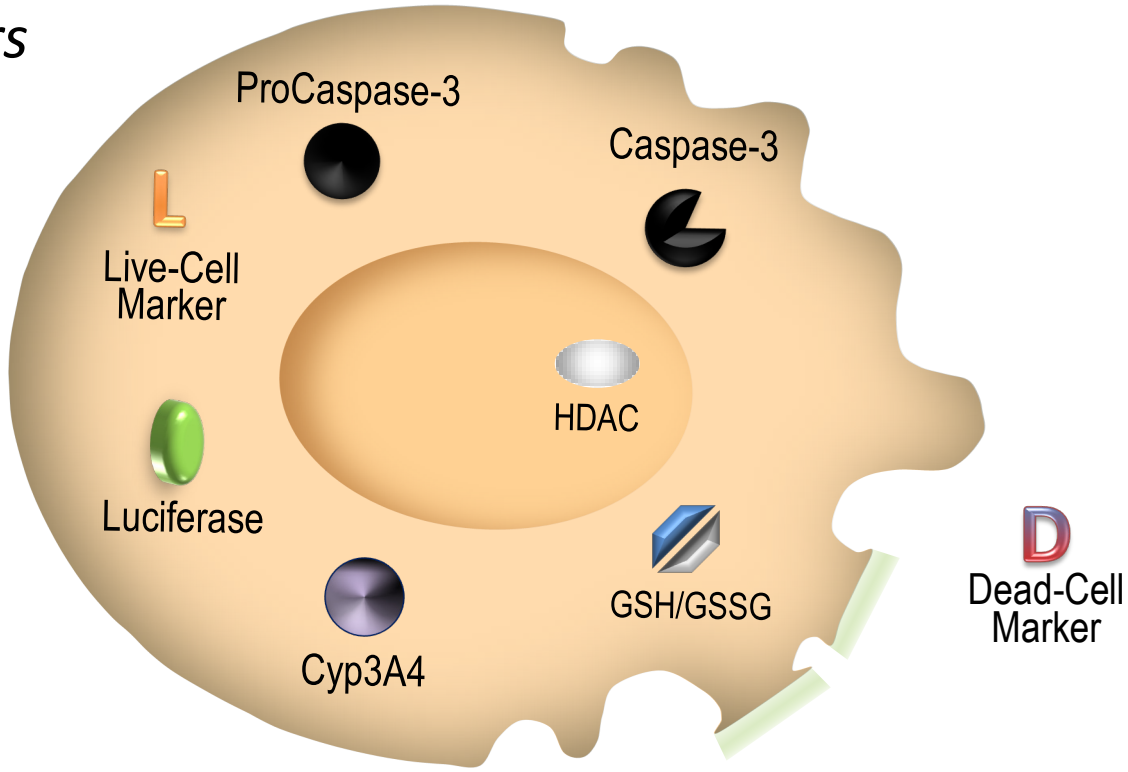
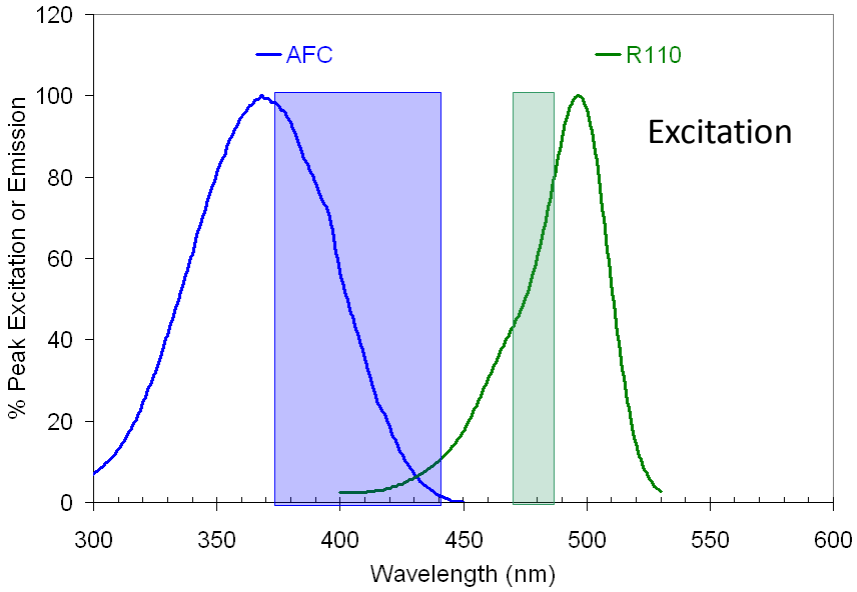
Detection Parameters

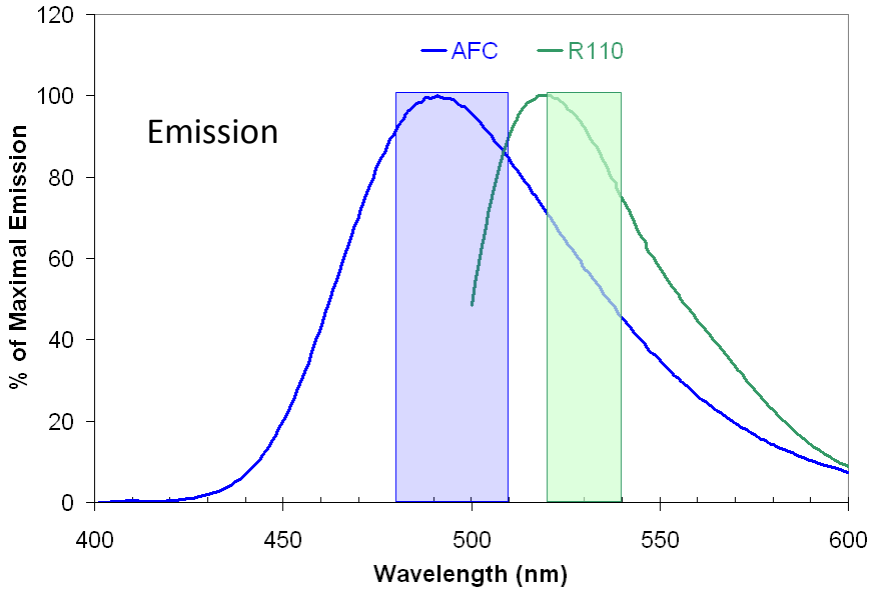


Detection Parameters – Filters & Fluorophores

The best filter settings for your multiplex may not equal the peak ex/em of the fluorophores…

- *Consider the spectral overlap of the multiplexed fluorophores*
- *Consider the capabilities of your detection platform*



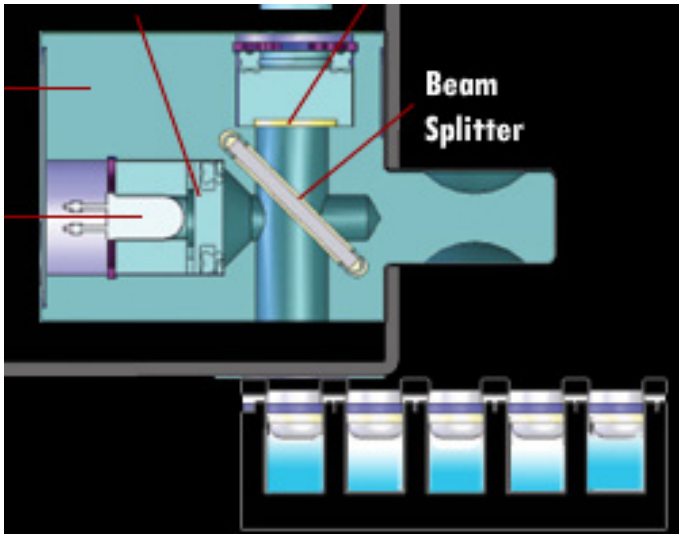


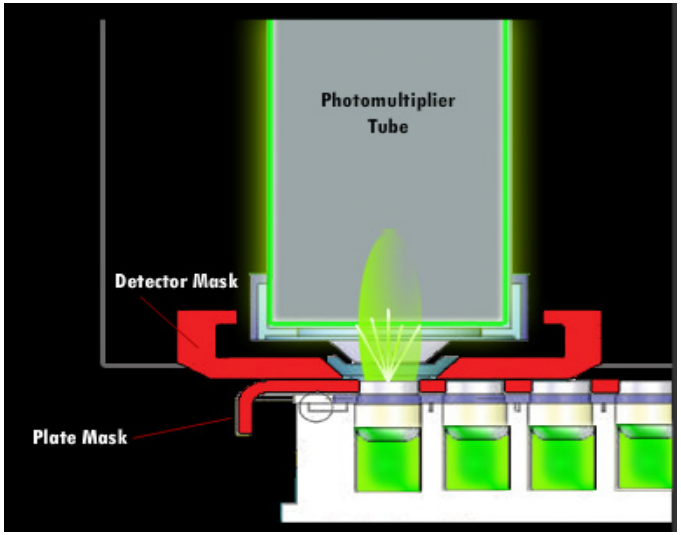
MutliTox-Fluor AFC & R110 dye spectra and example of compatible filter sets

Detection Parameters – Gain & Integration

- *Gain* – sensitivity setting
- *Integration* – time of signal collection

> - *User adjustable on many instruments*
> - *Settings too high or too low can cause problems*





Detection parameters affect assay performance

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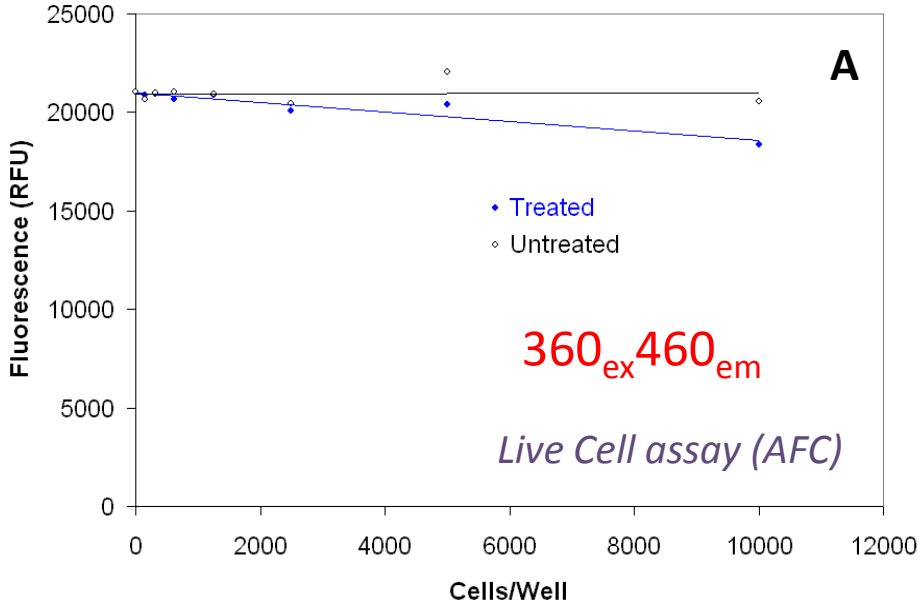
Symptoms:

No signal ↑ with cell # ↑;
no Δ with treatment

Cause:

Incorrect filters used!

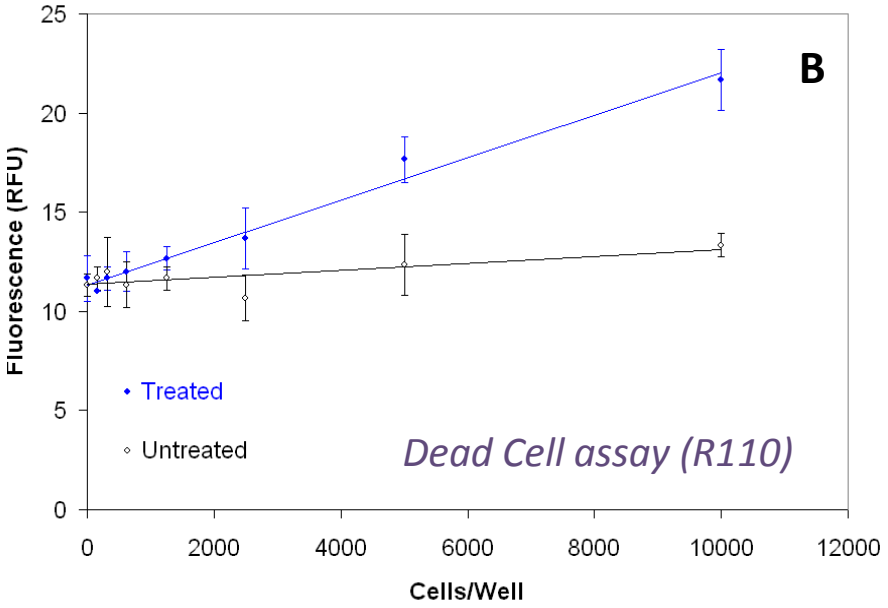


Symptoms:

Low signal, high variation;
little change with treatment

Cause:

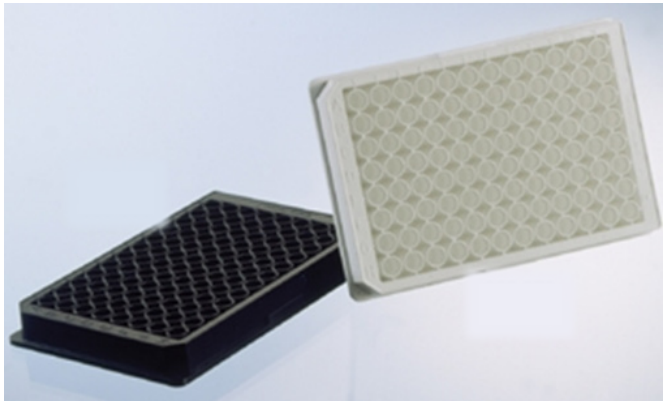
Gain is too low!



MultiTox-Fluor assay on serial dilutions of cells, +/- toxic treatment

Detection Parameters – plate choice

- *White or Black?*

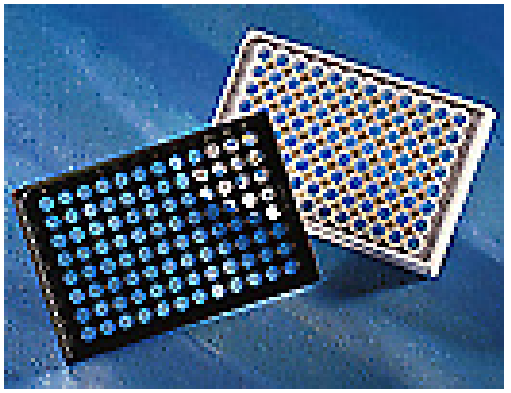


General recommendation:

- Black for fluorescence
- White for luminescence

- In reality, either plate can be used for both assays
- For multiplex we tend to use white

- *Solid or Clear Bottom?*



- Clear bottom plates can be used for viewing cultures prior to assay
- Opaque bottom gives better signal, less cross-talk

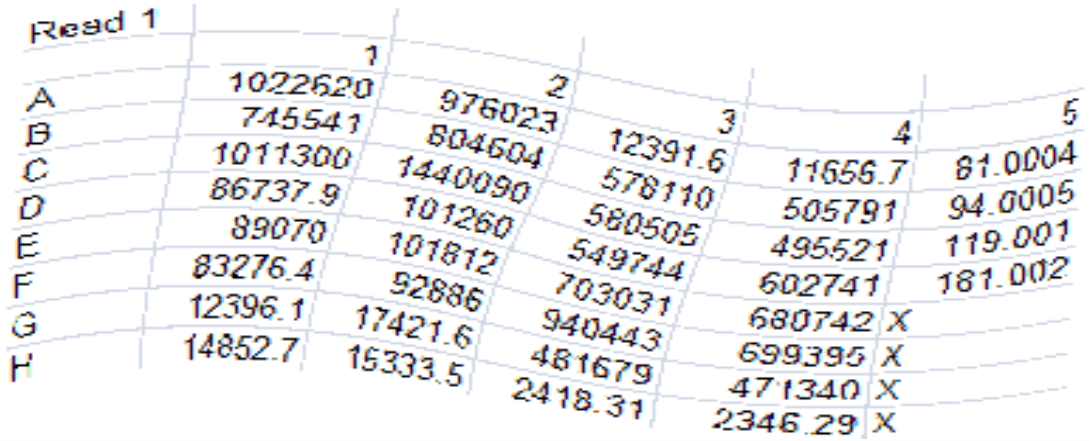
- Generally small effect on signal and cross-talk
- Opaque tape can be applied

Detection Parameters – Software/analysis issues

- The instrument software may be automatically…
  - *…subtracting background*
  - *…assigning & using a control sample for normalization*

  If so, are the correct wells being used?

What automatic functions are being applied?



Read 1	1	2	3	4	5
A	1022620	976023	12391.6	11656.7	81.0004
B	745541	804604	578110	505791	94.0005
C	1011300	1440090	580505	495521	119.001
D	86737.9	101260	549744	602741	181.002
E	89070	101812	703031	680742 X	
F	83276.4	92886	940443	699395 X	
G	12396.1	17421.6	461679	471340 X	
H	14852.7	15333.5	2418.31	2346.29 X	

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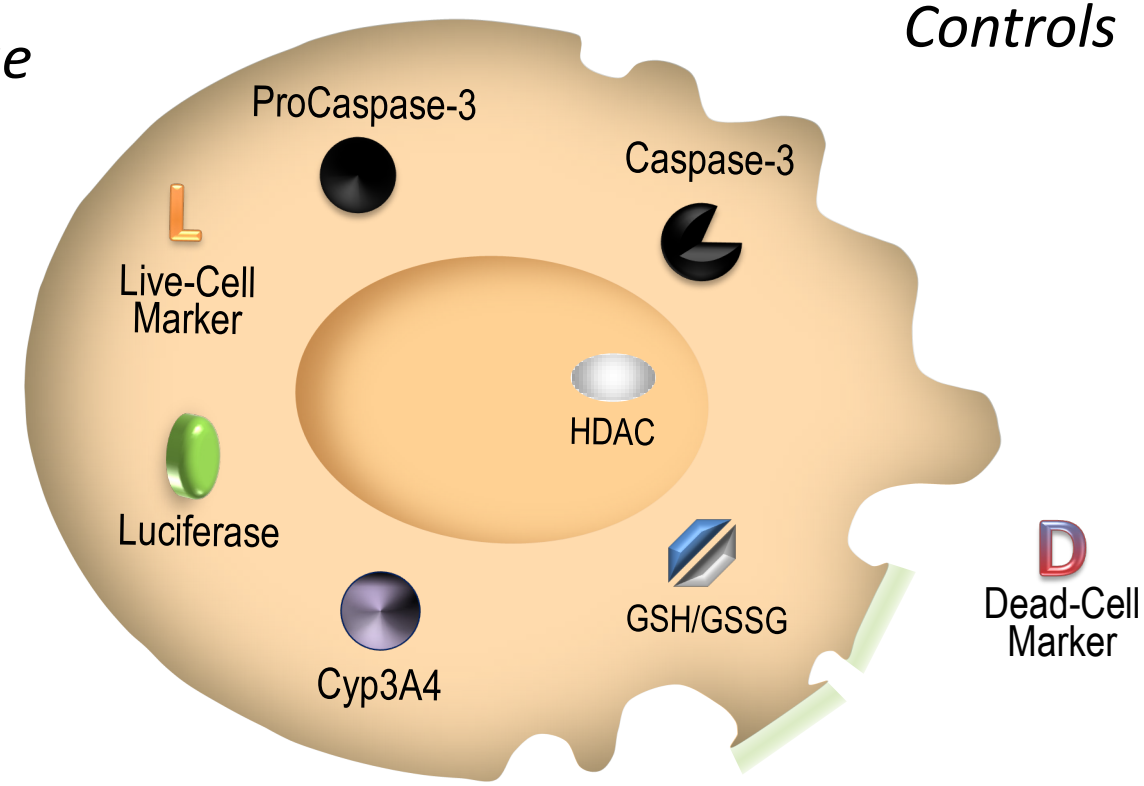
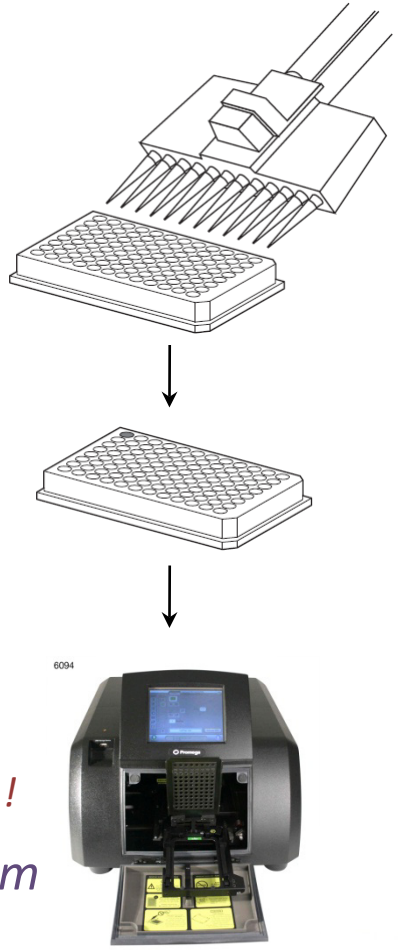
Assay Technique

Controls



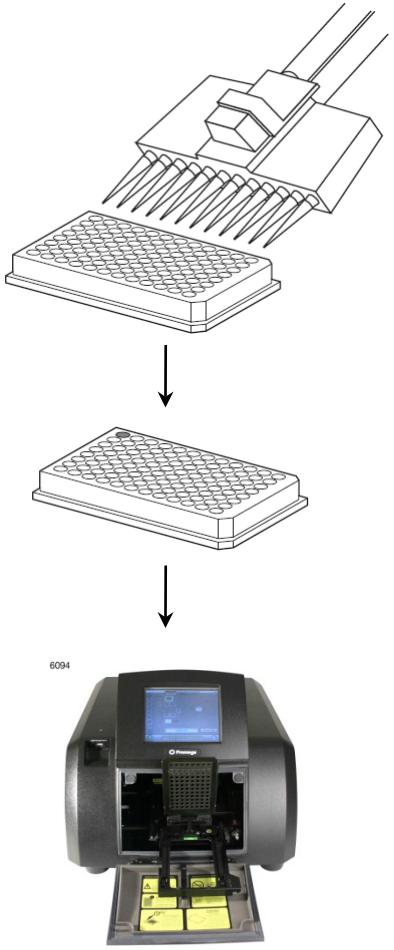
Common technical mistakes in cell-based assays

- Problems with reagent addition
  - *Introducing bubbles*
  - *Droplets of reagent on upper surfaces well*
  - *Insufficient mixing or inconsistent mixing*
- Poor temperature control during reagent incubation
  - *Enzyme assays are temperature dependent!*
  - Fluorogenic assays may be incubated at room temp or 37°C…
    - *either way, temperature should be consistent across plate*
    - *use of water bath is ideal*
    - *Beware cell culture incubators for 37°C reagent incubation!*
  - *Luminescence assays should always be incubated at room temperature (equilibrate reagent before addition)*



Common technical mistakes in cell-based assays

- Improper timing of reagent incubation
  - *Reading too soon may*
    - *↓ signal:noise*
    - *↓ sensitivity*
    - *↓ range*
    - *↓ discrimination*
  - *Incubating too long may*
    - *↓range – higher levels of analyte may saturate assay or detector*
  - *Optimal incubation period will vary with temperature*
    - *e.g. MultiTox-Fluor assays incubated at 37°C require ~30 minutes; incubation at room temperature may require up to 3 hours*



Assay controls

- No-Cell control
  - *Determines background of the assay (media/plate/reagent/detector)*
    - *Used in data processing, background subtraction*
- Untreated Cells Control
  - *Vehicle only*
    - *Also used in analysis - determining fold response, 100% viability &/or 0% cytotoxicity for relative response ratio*
- Positive Controls
  - *For cytotoxicity/apoptosis/pathway effect*
    - *Also used in analysis - determining fold response 0% viability and/or 100% cytotoxicity for relative response ratio*
- Multiplate assays
  - *Replicates of untreated control and a positive control*
    - *Controls for possible variation in processing, temp, timing, etc.*

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Type of Cell

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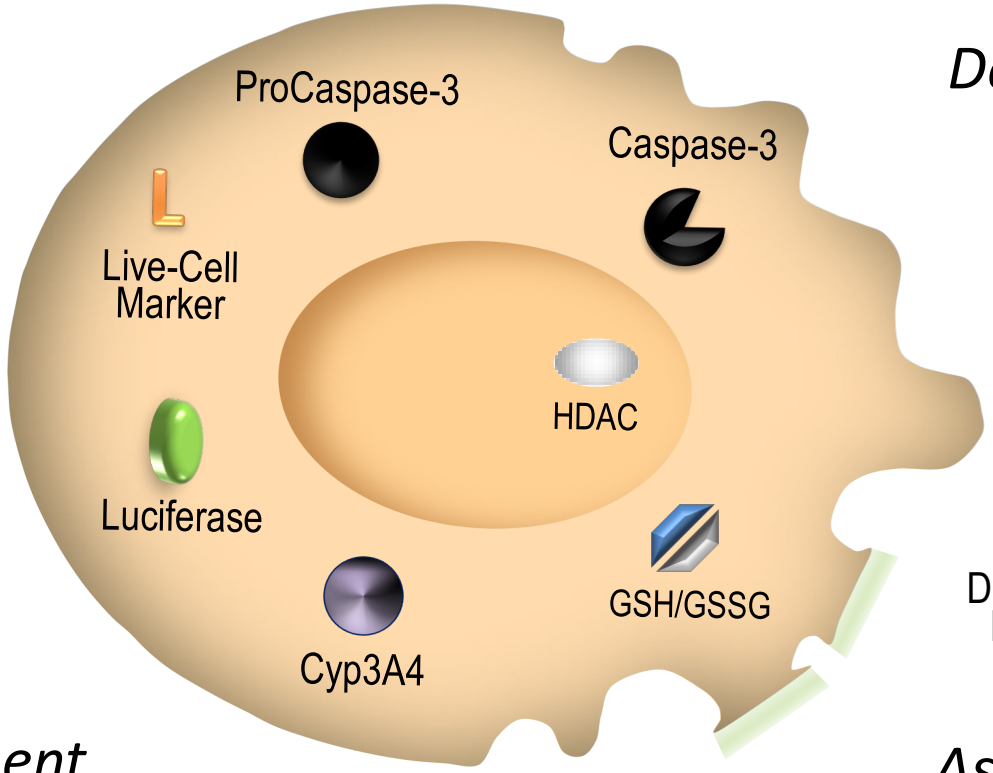

Questions?

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Rely on Promega Technical Services

- Experienced & highly trained scientists

  >150 years cumulative bench experience,

  >10 yrs average

- Varied technical expertise

  reporters, cell culture, HTS, etc.

- Varied scientific expertise

  model systems, genetics, development, etc.

- ***Easy!*** *– phone, chat, e-mail*