Chemical Genomics 101: Assays for HTS

NIEHS
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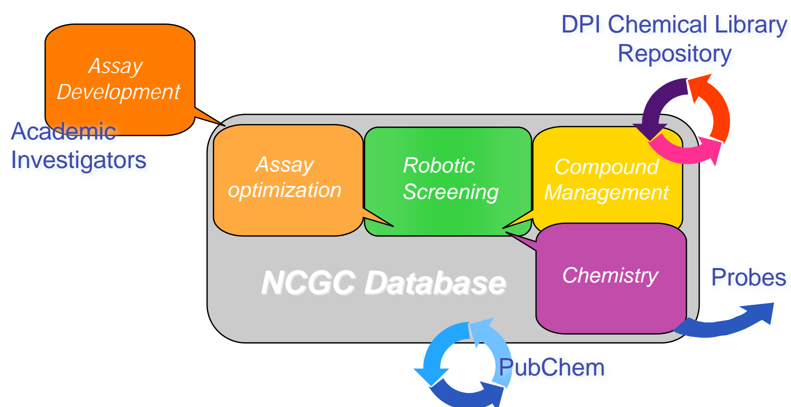
NIH Chemical Genomics Center
National Human Genome Research Institute
National Institutes of Health

Outline

- Assays and HTS
  - Definitions, plate formats, throughput, & volumes
  - Statistical measures of assay performance
  - Assays run at NCGC
- Assays: from bench top to robot
  - Example
- Assay artifacts

What is involved?

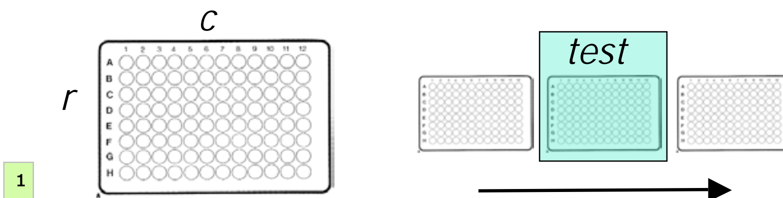
- Interfacing a biological assay with components of a HTS environment



Definitions

- HTS Assay: A precisely-defined and efficiently-designed experiment measuring the effect of a substance on a biological process of interest.

- High Throughput Screen (HTS): An iterative testing of different substances in a common assay. The testing occurs in parallel for a $r \times c$ array of assays sequentially for N arrays[1]. A screen is generally considered high throughput for >10,000 assays per day. Ultra HTS (uHTS) is generally reserved for >100,000 assays per 24 hrs.



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96, 384, and 1536-well microtiter plates

- 8 rows
- 12 columns (A-H)
- 88 test samples
- 8 controls (8.3%)

- 16 rows
- 32 columns (A-P)
- 352 test samples
- 32 controls (8.3%)

- 32 rows
- 48 columns (A-AF)
- 1,408 test samples
- 128 controls (8.3%)

Screening Throughput: Plate format dependence

- @ 200 microtiter plates per 24 hrs:

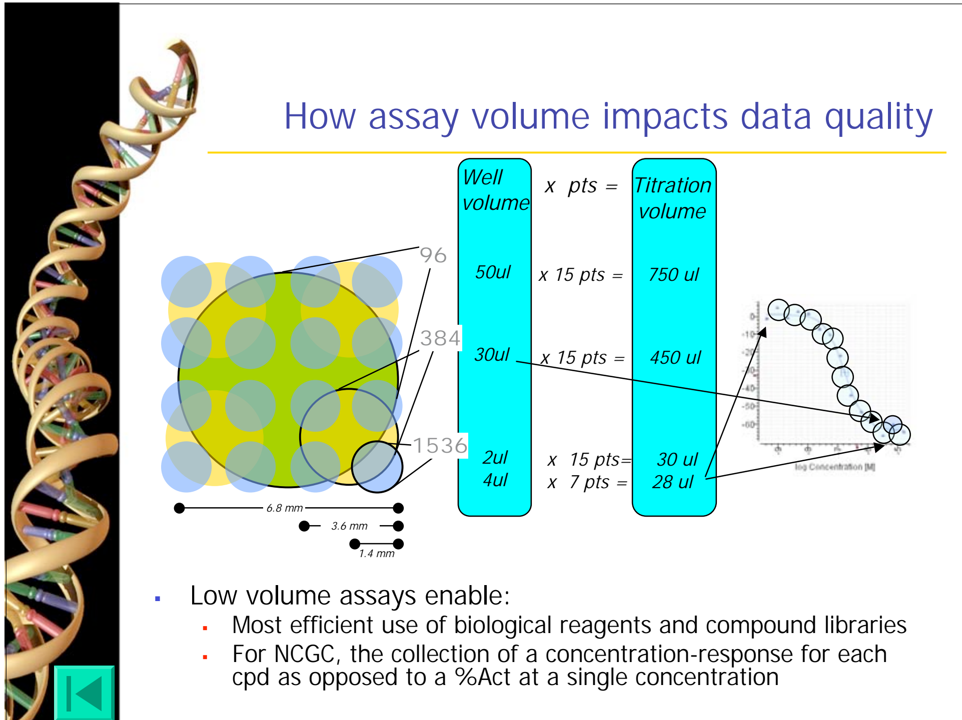
Plate format	samples§/day (wells/day)	Time to screen 1 MM samples
96-well	16,000 (19,200)	3.2 months
384-well	64,000 (76,800)	3½ weeks
1,536-well	281,600 (307,200)	3 ½ days
3,456-well	576,000 (691,200)	2 days

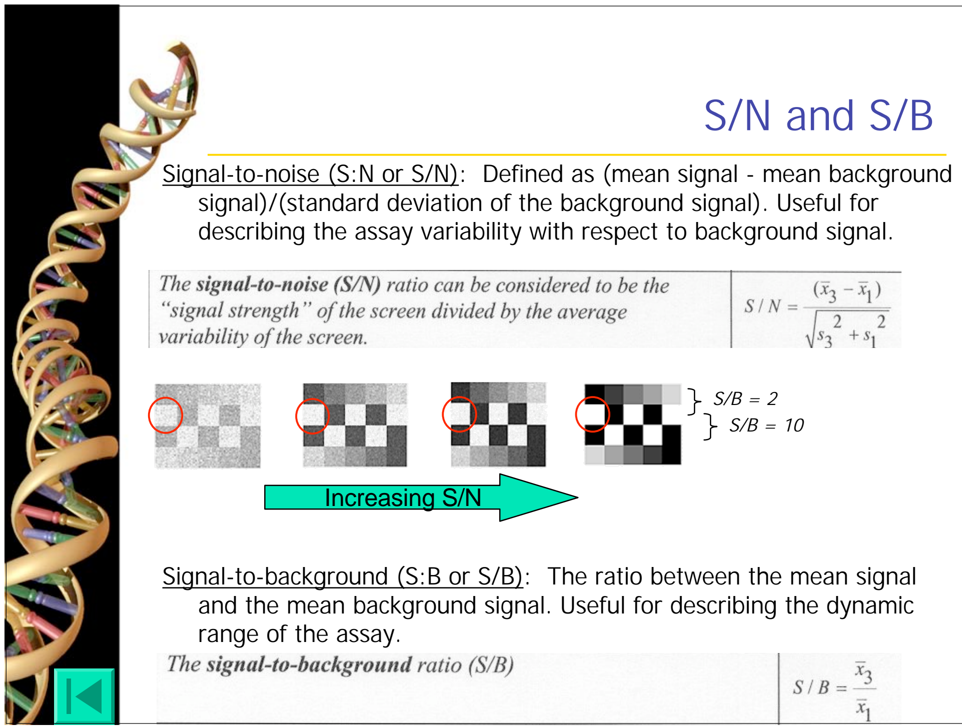
- §wells remaining after subtraction of control wells; NCGC uses left 4 columns of a 1536-well plate

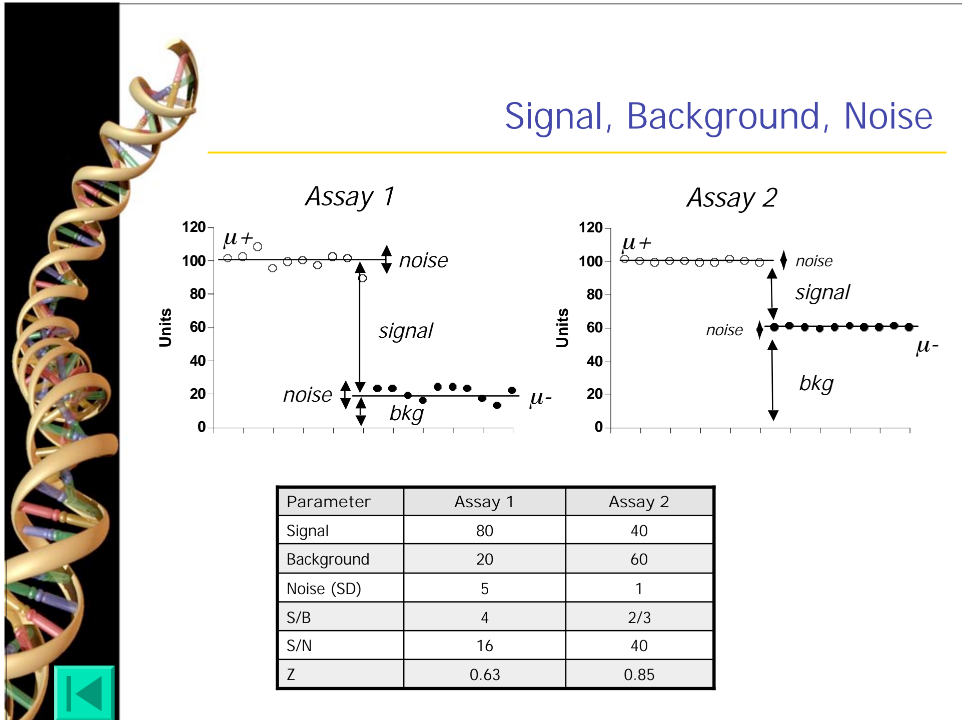
Cost to screen 100K samples

Eppendorf tube	*96-well plate*	*384-well plate*	*1536-well plate*
250 ul	*50 ul*	*25 ul*	*4 ul*
25 L	*5 L*	*2.5 L*	*400 mL*
$250,000	*$50,000*	*$25,000*	*$4,000 (4 cents/well)*

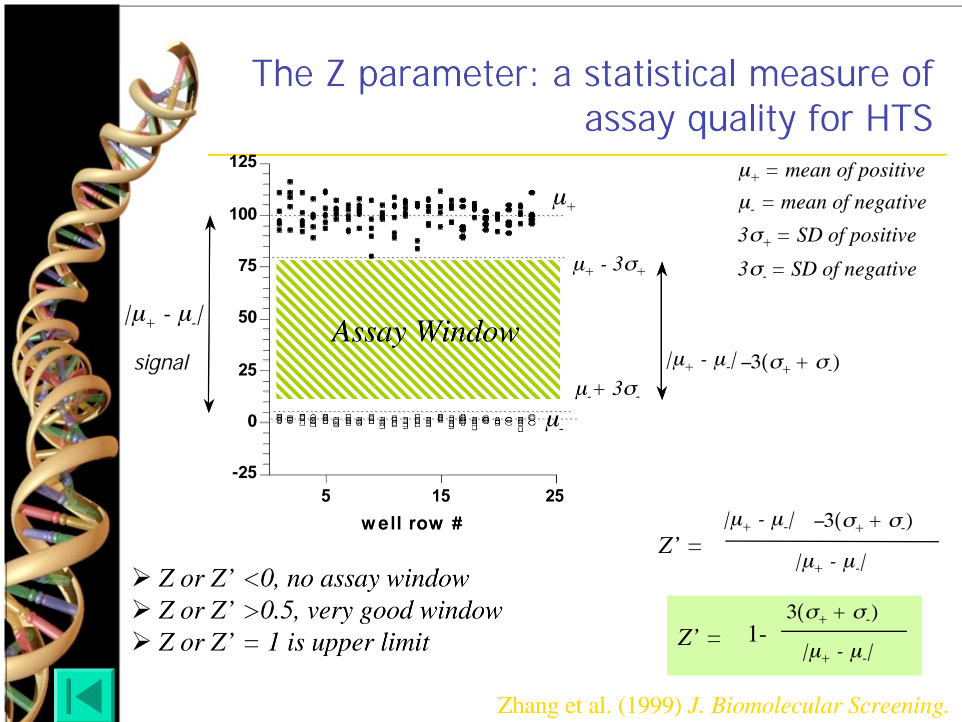
- 1536-well format combines cost-efficiency and assay versatility


How assay volume impacts data quality
Well volume
x pts =
Titration volume
96
384
1536
50ul
x 15 pts =
750 ul
30ul
x 15 pts =
450 ul
2ul
4ul
x 15 pts=
x 7 pts =
30 ul
28 ul
6.8 mm
3.6 mm
1.4 mm
Low volume assays enable:
Most efficient use of biological reagents and compound libraries
For NCGC, the collection of a concentration-response for each cpd as opposed to a %Act at a single concentration


S/N and S/B
Signal-to-noise (S:N or S/N): Defined as (mean signal - mean background signal)/(standard deviation of the background signal). Useful for describing the assay variability with respect to background signal.
The signal-to-noise (S/N) ratio can be considered to be the "signal strength" of the screen divided by the average variability of the screen.
$S/N = \frac{(\bar{x}_3 - \bar{x}_1)}{\sqrt{s_3^2 + s_1^2}}$
S/B = 2
S/B = 10
Increasing S/N
Signal-to-background (S:B or S/B): The ratio between the mean signal and the mean background signal. Useful for describing the dynamic range of the assay.
The signal-to-background ratio (S/B)
$S/B = \frac{\bar{x}_3}{\bar{x}_1}$

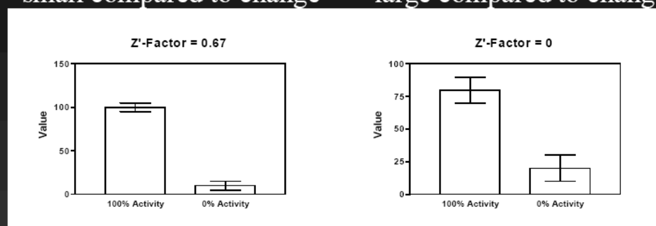


Parameter	Assay 1	Assay 2
Signal	80	40
Background	20	60
Noise (SD)	5	1
S/B	4	2/3
S/N	16	40
Z	0.63	0.85



Good range: Z’ = 0.5 to 1
Standard deviations are small compared to change

Bad range: Z’ < 0.5
Standard deviations are large compared to change



Source: Zhang et al. 1999, J. Biomolecular Screening 4: 67-73.

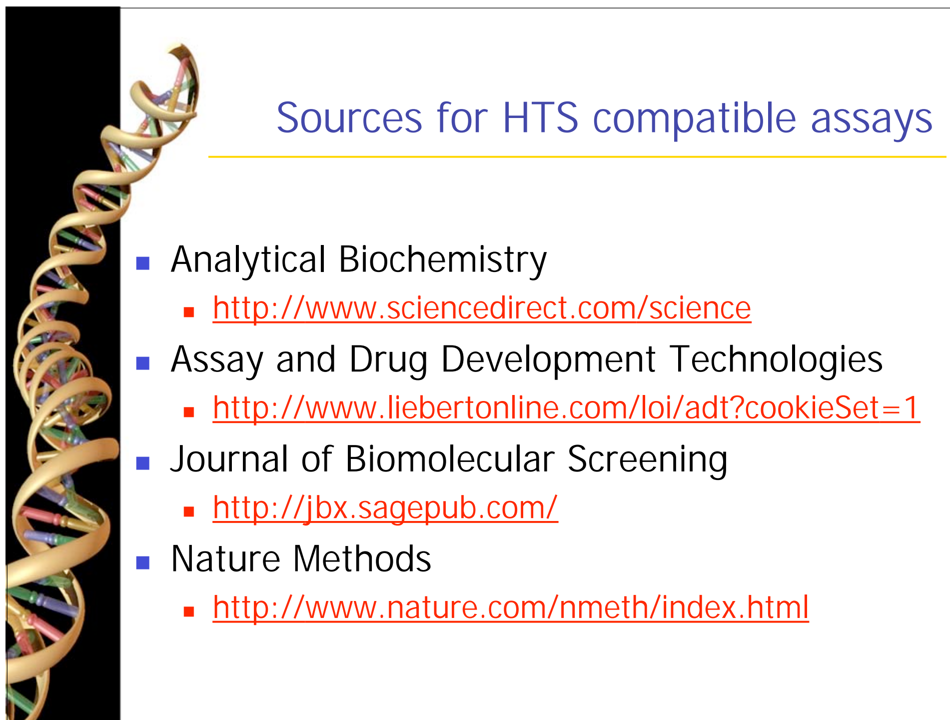
Helms,M., *Exelixis, Inc.* Presentation

- Assays and HTS
  - Definitions, plate formats, throughput, & volumes
  - Statistical measures of assay performance
  - Assays run at NCGC
- Assays: from bench top to robot
  - Example
- Assay artifacts

Criteria	Biochemical	Cell-based
*Plate Format **	96-well or higher density plate NCGC: 1536 -well format Assay volume 2 -6 ul	96-well or higher density plate NCGC: 1536 -well format Assay volume 4 -6 ul
Assay Steps	≤10 s teps with 96 -well plate. Steps include, reagent additions, timed incubations, plate transfers to incubator, reading, etc.	≤10 steps with 96 -well plate. Steps include, reagent additions, timed incubations, plate transfers to incubator, reading, etc.
Minimu m time increments and maximum assay duration	Minimum assay window is 5 min. (i.e., earliest time point after last reagent addition)	< 24 hr is ideal; max 48 hrs. Minimum assay window is 5 min.
Reagent Addition Steps	4 maximum (4 unique reagents max; more if pre - mixed)	4 maximum (4 unique reagents including cells max; more if pre -mixed)

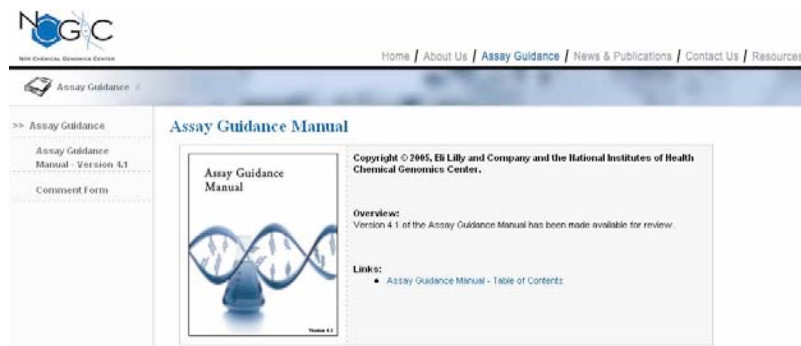
Criteria	Biochemical	Cell-based
Temperature	Between RT and 37°C	Between RT and 37°C
*Demonstrated DMSO Tolerance **	0.5 – 1% DMSO	0.5 -1% DMSO
Signal : Background Ratio	≥ 3 -fold	≥ 3 -fold
Day-to-Day variation of control (e.g., IC_{50}, EC_{50})	< 3-fold	< 3-fold
Reagent stability @ final working concentration	≥ 8 hrs @ RT or on ice bath; No on -line thawing	≥ 8 hrs @ RT or on ice bath; No on -line thawing
Validation run reagent supply	10 – 96 -well plate equivalents	10 – 96 -well p late equivalents
Protocol	Complete detailed protocol. All steps, equipment used, all vendor & catalog # for reagents. Data from 96 -well or high density plate tests.	Complete detailed protocol. All steps, equipment used, all vendor & catalog # for reag ents. Detailed cell culture procedure, passage # .Data from 96 -well or high density plate tests.

Criteria	Biochemical	Cell-based
NCGC Detectors (assay must be able to be read on one of these detectors)	**PE ViewLux** (Top reading only: FI, TRF, FP, Abs, Luminescence) **PE Envision** (bottom reading FI, ALPHA) **Acumen Explorer** (fluorescent laser cytometry) (laser: 488 nm Ar -ion)	**PE ViewLux** (Top reading only: FI, TRF, FP, Abs, Luminescence) **PE Envision** (bottom reading FI, ALPHA) **Acumen Explorer** (fluorescent laser cytometry) (laser: 488 nm Ar -ion)
Examples of assay formats used at NCGC	Fluorescent Intensity NADPH oxid. (Ex360 / Em450 nm) Pro-fluorescent substrates Resorufin (Ex 570 / Em 590 nm) 4-Methylumbelliferone (365/440) Fluorescence Polarization Fluorescein -labeled DNA Fluorescein -UDP-GlcNAc Luminescence Luciferase -coupled Absorbance	Reporter assays Luciferase / Luciferin Beta-lactamase / CCF4 GFP expression Cell Sensor assays Dual Luciferase s (Red & Green) GFP complementation assay Cell viability assays ATP Glow Translocation assays GFP-HNR fusion: cytosol to Nuc. EFC-HNR fusion: cytosol to Nuc.
Special	For unique reagents either investigator prepares sufficient quantity for HTS or identifies a reliable 3rd party vendor.	Cells must be certified micoplasma -free by direct culture as say and cell -DNA fluorochrome staining.



NCGC website

- *Assay Guidance Manual Version 4.1*, 2005, Eli Lilly and Company and NIH Chemical Genomics Center.
  - http://www.ncgc.nih.gov/guidance/index.html



Additional Links

- Introduction to Fluorescence Techniques
  - http://probes.invitrogen.com/handbook/sections/0001.html
  - Fluorescence Spectra Viewer
    - http://probes.invitrogen.com/resources/spectraviewer/
- Reporter Gene Technology
  - β– Lactamase as a Gene Reporter System
    - http://www.bio.davidson.edu/courses/Molbio/MolStudents/spring2000/rice/method.html

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- Assays: from bench top to robot
  - Examples
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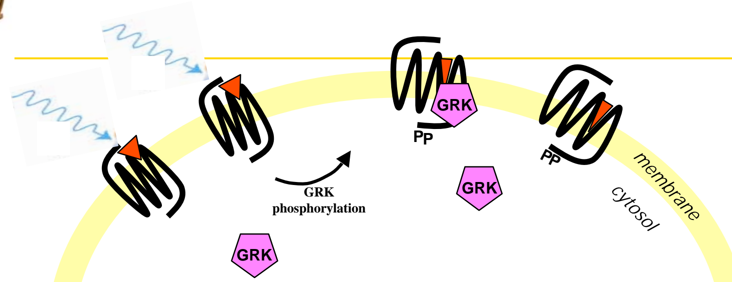
Bench top to Robot 1

- GRK example

Assay-HTS Design

- Enzymology vs. cell biology
  - Importance of biological context
- Assay
  - Reagents (few or many experiments)
  - Cost
- Endpoint(s)
  - Detector
    - Radioactivity
    - Fluorescence / Luminescence
      - Endpoint (reporter gene)
      - Rapid kinetics, (e.g. Ca^{2+}-flux)
    - Imaging microscopy (GFP-target)
    - Other

G protein-coupled receptor kinase assay

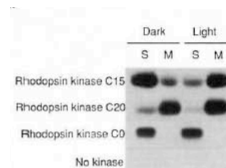


- Develop an assay for the putative regulatory kinases (GRKs) of the β-adrenergic receptor (β-AR)
  - No suitable peptide substrates available; receptor:kinase complex was of interest
  - ∴Needed to use physiological substrate, transmembrane spanning receptor
  - β-AR was difficult to prep. in sufficient quantity
  - ∴Needed to use a more abundant superfamily member, rhodopsin
  - GRKs expressed in COS cells
  - Reconstitute membrane receptor kinase system

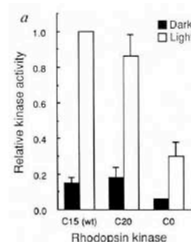
Journal "Materials and Methods" Protocol

Translocation-Phosphorylation Assay

- *measures the "location" of kinase*
- *measures the phosphorylation state of R*



. Translocation assays were done in polycarbonate centrifuge tubes (Beckman 8 × 34 mm) each tube containing a total volume of 40 μl translocation buffer (50 mM Na_2HPO_4, pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$, 5 mM DTT, 10 μM protein kinase A inhibitor and protease inhibitors) and 20 μM rhodopsin (urea-treated ROS membranes). Diluted kinase supernatants were added and one tube of each pair was kept in the dark and the other illuminated with fluorescent white light at 25 °C for 3 min. Both tubes were then centrifuged at 350,000*g* for 5 min at 4 °C. Supernatnats were quickly removed with 50 μl Hamilton syringe under room light. Bleached rhodopsin was added to a final concentration of 20 μM. Pellets were resuspended in translocation buffer plus one volume of ROS dilution buffer (5 mM Tris-HCl, pH 7.4, mM DTT, 2 mM $MgCl_2$, 65 mM NaCl) to equalize the sample volumes. Four samples (dark/supernatant, dark/membrane, light/supernatant, light/membrane) were assayed for kinase activity (as described previously) or immunoblotted.



Inglese, Koch, Caron, & Lefkowitz, 1992 *Nature* 359

Methods in Enzymology Protocol

Eppendorf tube on ice

Vortex the mixture

pipette up and down

polycarbonate tubes

quickly remove supernatants

resuspend the pellet

[³²P]ATP

followed by SDS-PAGE

X-ray film

3. To a 1.5-ml Eppendorf tube on ice is added 18 μl of translocation buffer (5×), 34 μl water, 20 μl of kinase or cell extract containing expressed kinase, and 18 μl of ROS membranes (stock concentration ~4 mg/ml rhodopsin, ~100 μM). Vortex the mixture or pipette up and down to ensure that the ROS membranes are suspended. To a separate 1.5-ml Eppendorf tube add 18 μl of ROS membranes and set the tube aside.

4. To a pair of polycarbonate centrifuge tubes (Beckman, Palo Alto, CA, 8 × 34 mm) add 40-μl aliquots from the mixture in Step 3. One tube (labeled −hν) is kept in the dark at ambient temperature while the second tube (labeled +hν) is illuminated with fluorescent white light at 25° (ambient temperature) for 3–5 min (a clear plastic Eppendorf rack over a light box works well to hold the tube during this step). At this time also illuminate the second Eppendorf tube of ROS membranes from Step 3.

5. After illumination return the samples to the darkroom, load the polycarbonate tubes (illuminated and dark samples) into a Beckman TLA [illegible], and centrifuge at 100,000 rpm (~350,000 *g*) for 5 min at 4° in [illegible] TL-100 ultracentrifuge to pellet the ROS membranes.[31]

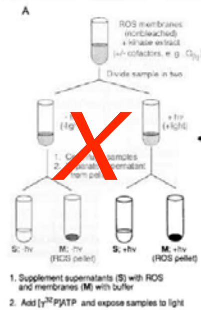
[illegible] centrifugation quickly remove supernatants (S) with a 50-μl [illegible] syringe (No. 805RN with a 25-gauge blunt-end needle) under room light and place in separate 1.5-ml Eppendorf tubes labeled S,−hν and S,+hν. To these supernatants add 8 μl of bleached rhodopsin from Step 4 to give a final concentration of 20 μM as in the precentrifuged mixtures. Using the 50-μl Hamilton syringe resuspend the pelleted membranes (M,−hν and M,+hν) in 40 μl of translocation buffer (1×) plus 8 μl of ROS membrane dilution buffer (50 mM Tris-HCl, pH 7.4, 1 mM DTT, 2 mM $MgCl_2$) to equalize the sample volumes.

7. To each of the four samples (−hν,S; −hν,M; +hν,S; and +hν,M) add 5 μl of ATP cocktail. ATP cocktail for four samples contains 3.2 μl of 10 mM ATP, around 1 μl [γ-³²P]ATP (NEG-002A, NEN-DuPont, Boston, MA; ~10 μCi), 2.5 μl of 10 mM $MgCl_2$, and 18.3 μl water. This will give a final [ATP] of 100 μM. Mix samples on addition of ATP cocktail by pipetting up and down. Allow the phosphorylation reaction to proceed for about 5 min (longer or shorter times may be necessary depending on the kinase activity) at ambient temperature or in a water bath at 30° illuminated by white light.

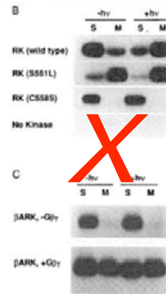
8. The reactions can be quenched and analyzed by addition of 10–20 μl of SDS sample buffer followed by SDS–PAGE on a 12% polyacrylamide gel. Allow the ATP to run off the gel (i.e., let tracking dye run off gel) or cut off the bottom of the gel before drying.[32] The dried gel can be either exposed to X-ray film or analyzed using a phosphor imager. An alternative method to quenching samples from this type of phosphorylation assay that helps reduce signal-to-noise ratios and remove ATP is described by Benovic.[18]

What's wrong with this picture?

Reagents & materials



Detection



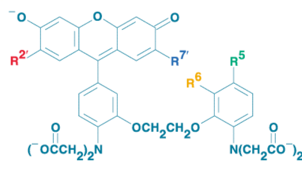
Protocol

- Eppendorf tubes
- Vortex
- Centrifuge tubes
- Light sensitive materials
  - Light box
  - Dark room
- Separation
  - Hamilton Syringe
- Re-suspension
- SDS-PAGE separation
  - Dry gel
- Expose to x-ray film
- Densitometer

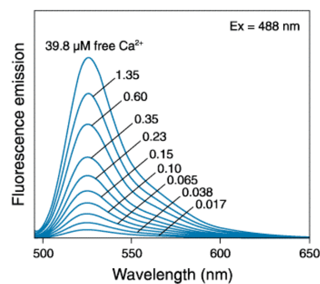
Also…

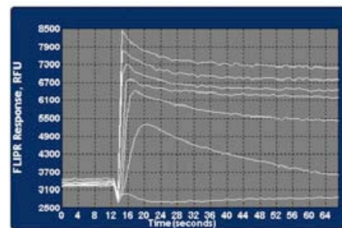
- Model system
- Regents are hard to obtain, handle
- Cellular context lost

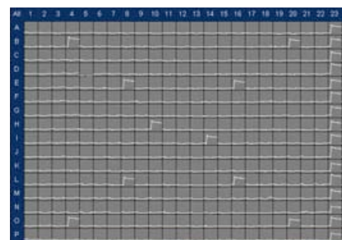
Measuring the function of GPCRs: Second messengers



Indicator	$K_d(Ca^{2+})$	$R^{2'}$	$R^{7'}$	R^5	R^6
Fluo-3	0.39 μM	Cl	Cl	CH_3	H
Fluo-4	0.35 μM	F	F	CH_3	H

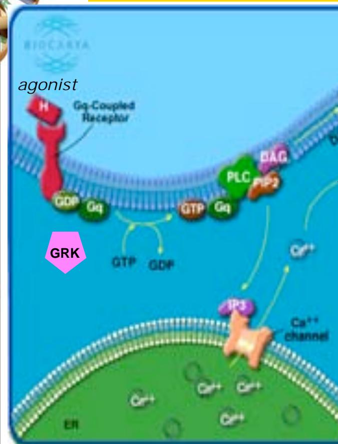


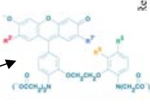


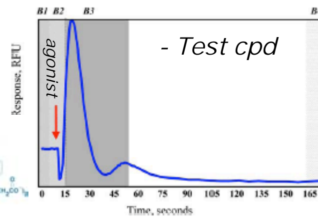


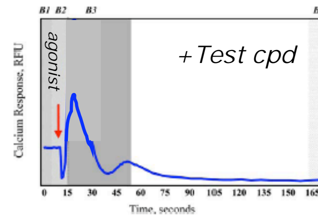
http://probes.invitrogen.com/

FLIPR desensitization fluo-3AM assay



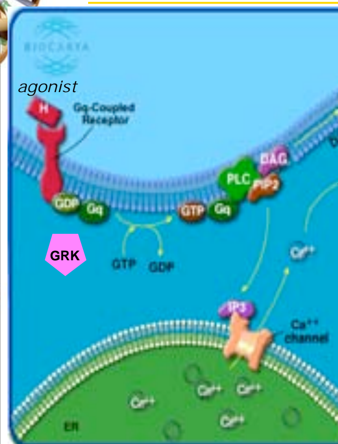


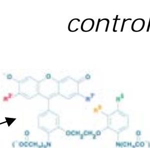




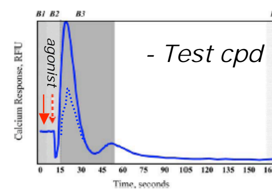
- Second messenger measurements are well established in HTS (cAMP, Ca^{2+}, IP)

FLIPR desensitization fluo-3AM assay

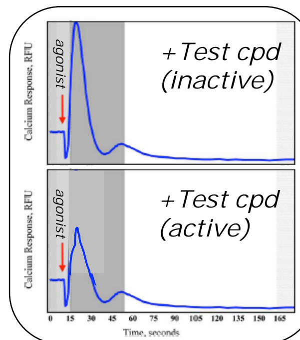




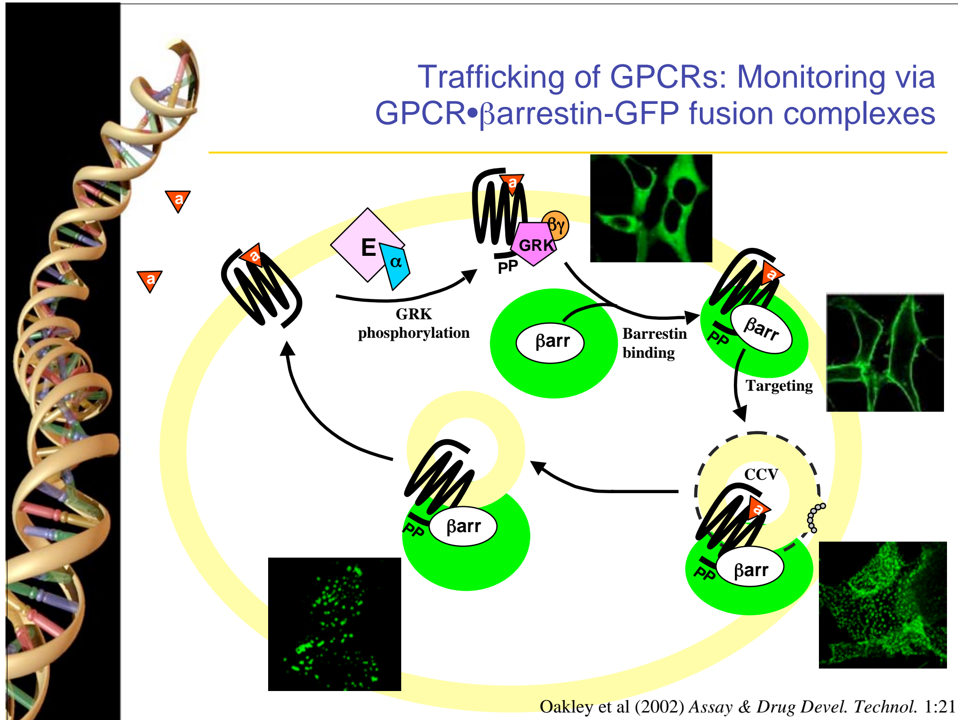
controls



screen



Trafficking of GPCRs: Monitoring via GPCR•βarrestin-GFP fusion complexes



Oakley et al (2002) *Assay & Drug Devel. Technol.* 1:21

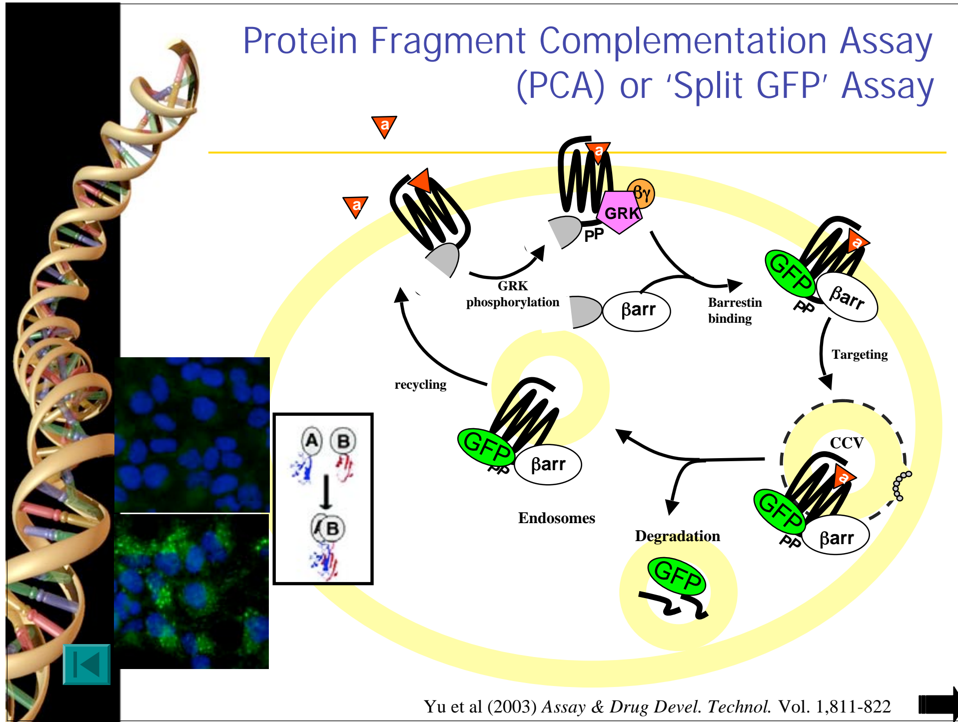
Imaging Microscopy Systems

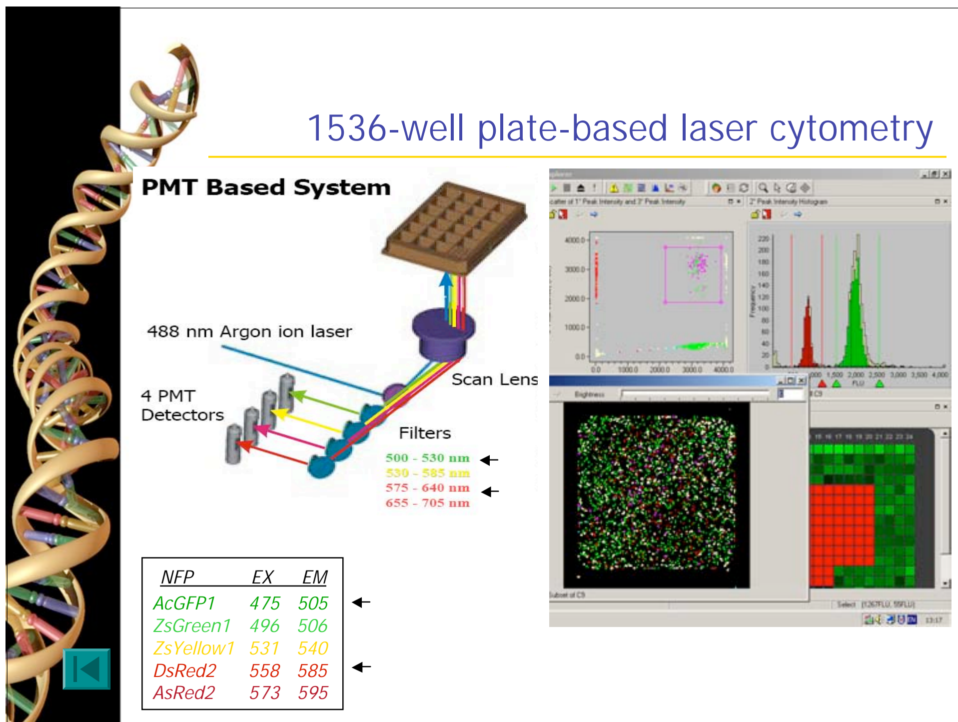
- Widefield
  - light collected from entire depth of sample
  - light source: Hg or Xe lamp
    - many EX λ possible
    - but, light intensity is limited
  - Commercial Systems
    - Cellomics ArrayScan
    - Q3DM EIDAQ 100
    - Amersham IN Cell 1000
      - (Imaging Research)
    - Molecular Devices Discovery 1
      - (Universal Imaging)
    - Axon ImageXpress

- Scanning Confocal
  - use pinhole or slit to eliminate out-of-focus light from detector
  - laser illumination
    - EX λ limited to available laser lines
    - high intensity EX
  - Commercial systems
    - Amersham IN Cell 3000

- Spinning Disk Confocal
  - rotating array of microlenses to focus illumination & 2nd array of rotating pinholes provides confocality.
    - Speed due to multifocal imaging
    - Reduced photo damage
  - Commercial System
    - Evotec Technologies OPERA

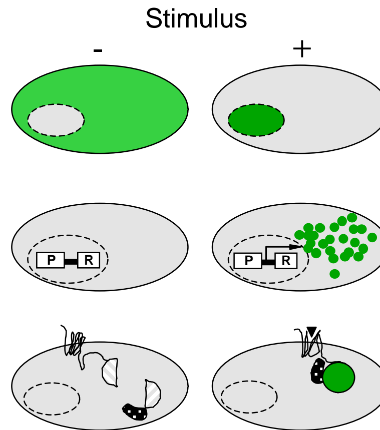
4 April 2003 Science


Protein Fragment Complementation Assay (PCA) or 'Split GFP' Assay
GRK
phosphorylation
βarr
Barrestin binding
Targeting
recycling
CCV
Endosomes
Degradation
GFP
Yu et al (2003) Assay & Drug Devel. Technol. Vol. 1,811-822


1536-well plate-based laser cytometry
PMT Based System
488 nm Argon ion laser
Scan Lens
4 PMT Detectors
Filters
500 - 530 nm
530 - 585 nm
575 - 640 nm
655 - 705 nm
NFP EX EM
AcGFP1 475 505
ZsGreen1 496 506
ZsYellow1 531 540
DsRed2 558 585
AsRed2 573 595

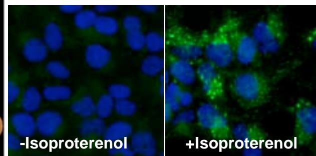
Fluorescent Protein-based Assays

- Protein Translocation
  - Cytoplasmic to Nuclear
    - GR-GFP
- Protein Expression
  - GFP reporter
- Protein Complementation
  - GPCR trafficking
  - others

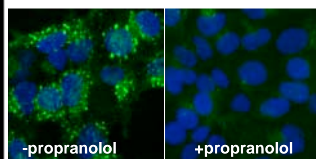


Phenotypic Assay: 1536-well qHTS Protocol

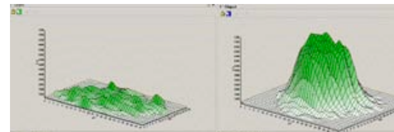
- Validation: Well-characterized biology
- Conditions for observing both agonists and antagonists worked out.
- Potentially useful in developing assays for proteins of unknown or poorly understood function



Test cpd is agonist

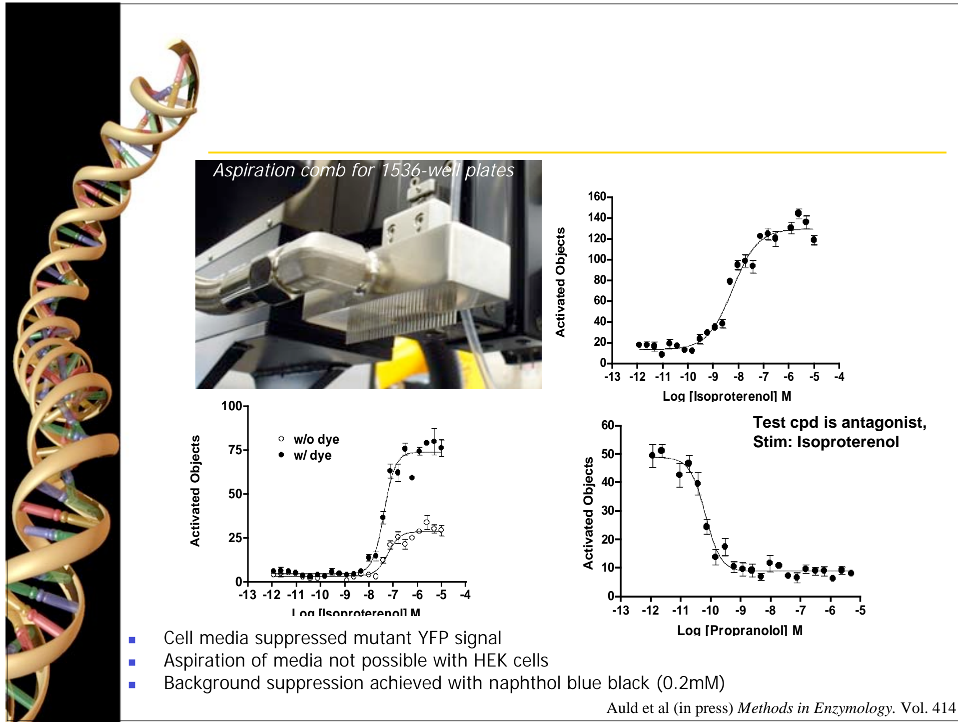


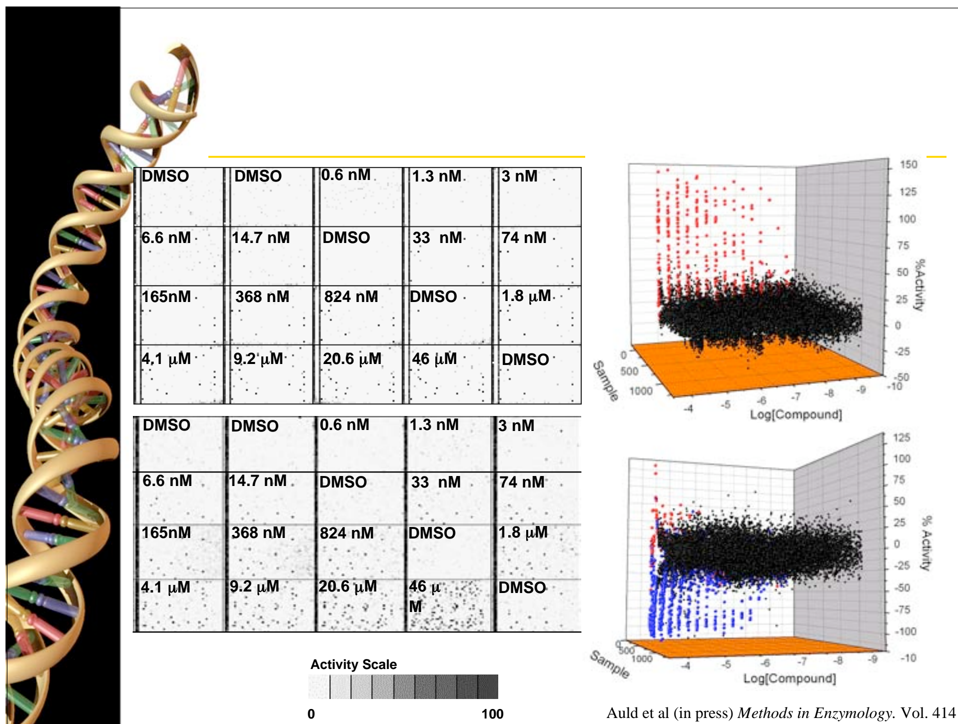
Test cpd is antagonist, +Isoproterenol

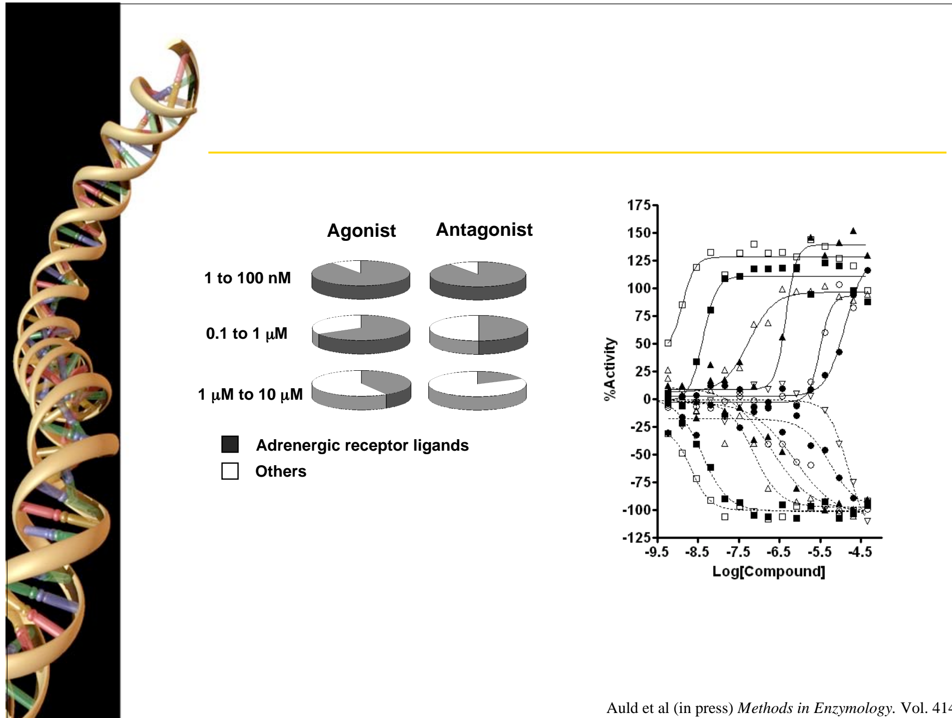


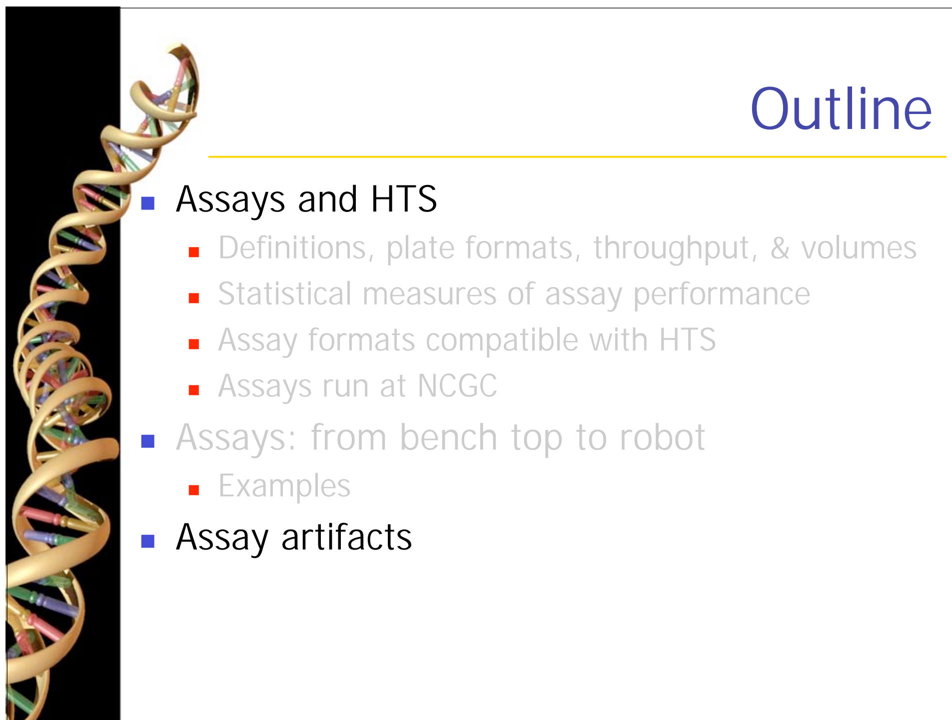
Split-GFP GPCR Activation Assay

Sequence	Parameter	Value	Description
1	Reagent	5 µL	Cells- 700/well
2	Time	24 hr	37° incubation
3	Cpmd	20 nL	40 µM – 0.5 nM
4	Time	1.5 hr	37° incubation
5	Reagent	1 µL	Queching dye
6	Detector	488nm /GFP	Acumen Explorer


Aspiration comb for 1536-well plates
Activated Objects
Log [Isoproterenol] M
w/o dye
w/ dye
Test cpd is antagonist,
Stim: Isoproterenol
Log [Propranolol] M
Cell media suppressed mutant YFP signal
Aspiration of media not possible with HEK cells
Background suppression achieved with naphthol blue black (0.2mM)
Auld et al (in press) Methods in Enzymology. Vol. 414


DMSO
0.6 nM
1.3 nM
3 nM
6.6 nM
14.7 nM
33 nM
74 nM
165nM
368 nM
824 nM
1.8 μM
4.1 μM
9.2 μM
20.6 μM
46 μM
Activity Scale
0
100
%Activity
Sample
Log[Compound]
Auld et al (in press) Methods in Enzymology. Vol. 414

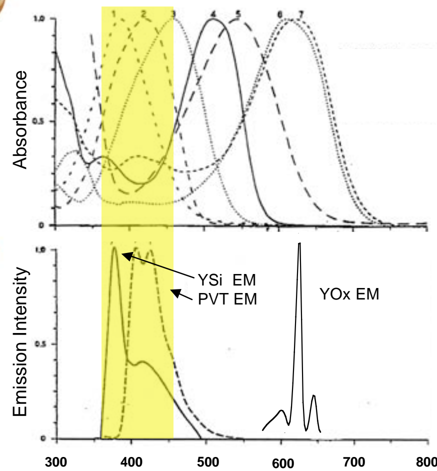

Agonist
Antagonist
1 to 100 nM
0.1 to 1 μM
1 μM to 10 μM
Adrenergic receptor ligands
Others
%Activity
Log[Compound]
Auld et al (in press) Methods in Enzymology. Vol. 414

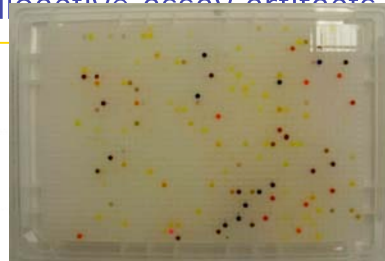

Outline
Assays and HTS
Definitions, plate formats, throughput, & volumes
Statistical measures of assay performance
Assay formats compatible with HTS
Assays run at NCGC
Assays: from bench top to robot
Examples
Assay artifacts

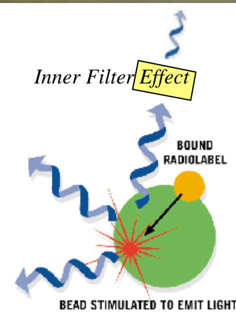
Artifacts

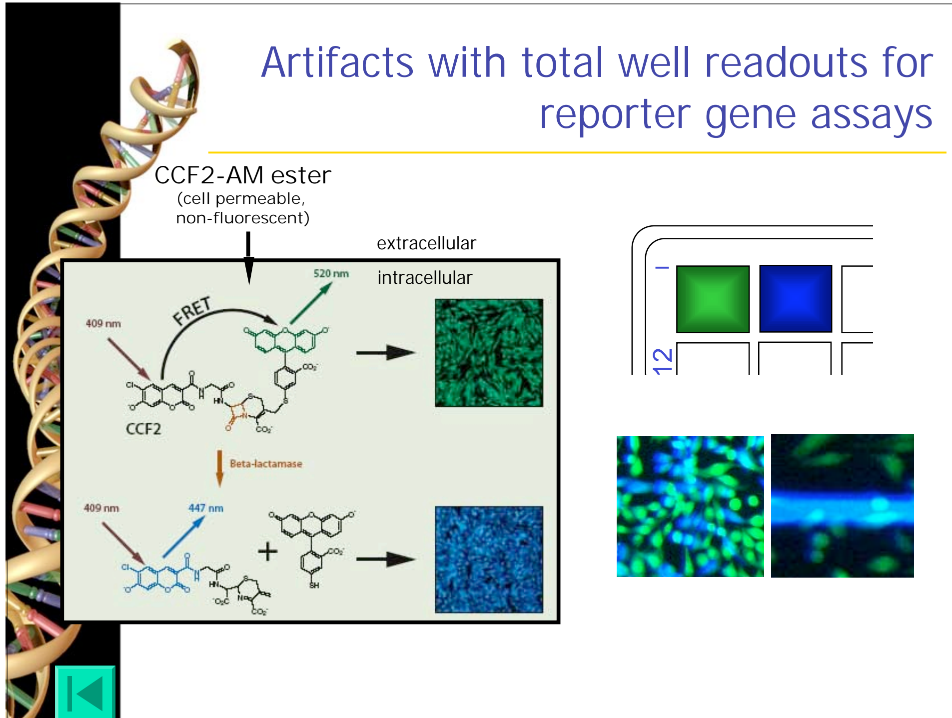
- Artifacts
- Inner filter effect
- "Blue lint"
- Fluorescent "bloom"
- Aggregation

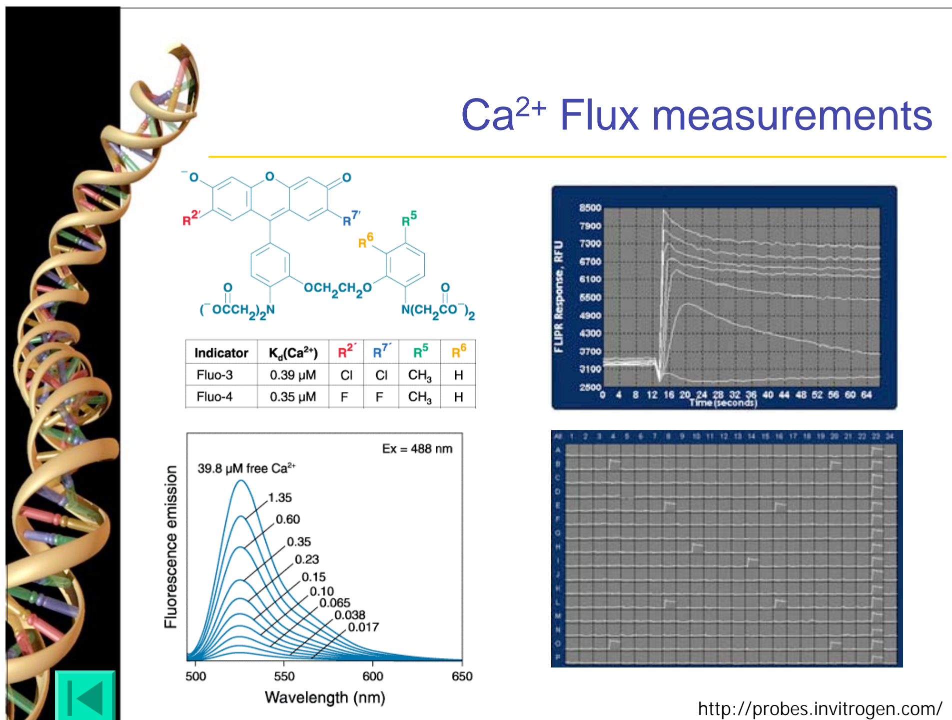
Inner Filter effect: luminescence, absorbance and radioactive assay artifacts



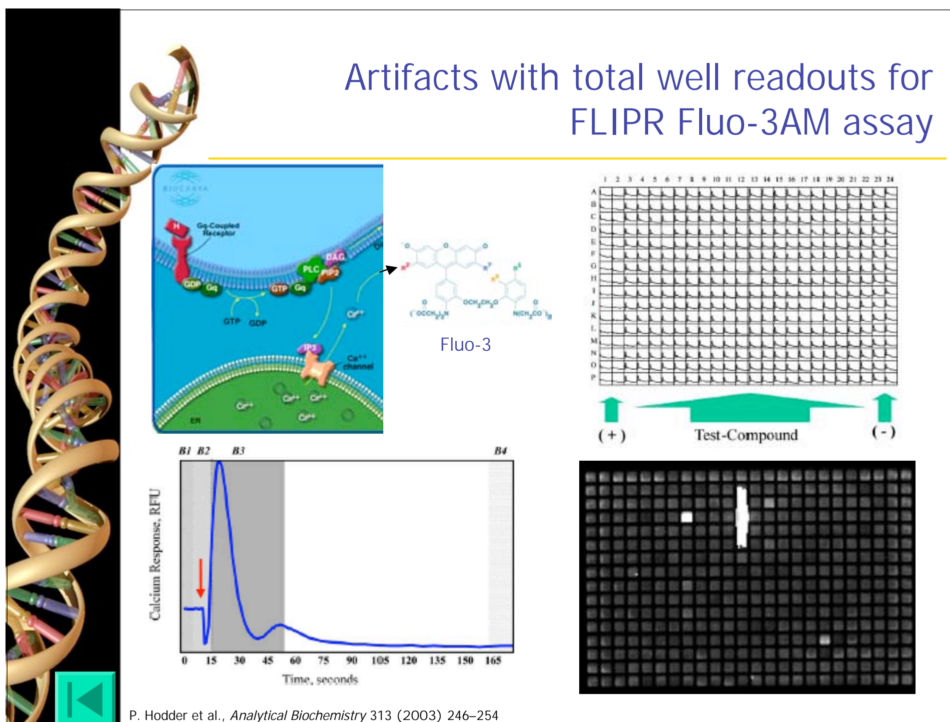





Artifacts with total well readouts for reporter gene assays
CCF2-AM ester
(cell permeable, non-fluorescent)
extracellular
intracellular
520 nm
FRET
409 nm
CCF2
Beta-lactamase
409 nm
447 nm
1
12


Ca2+ Flux measurements
Indicator
Fluo-3
Fluo-4
0.39 µM
0.35 µM
Ex = 488 nm
39.8 µM free Ca2+
Fluorescence emission
Wavelength (nm)
FLIPR Response, RFU
Time (seconds)
http://probes.invitrogen.com/

Artifacts with total well readouts for FLIPR Fluo-3AM assay



P. Hodder et al., *Analytical Biochemistry* 313 (2003) 246–254

Promiscuous Inhibitors

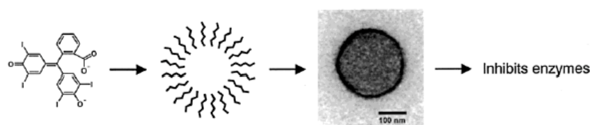


Figure 3. Summary of the proposed mechanism of nonspecific inhibition. Some small molecules form particles from 50 to over 400 nm in diameter, and these particles adsorb or absorb target enzymes, thereby inhibiting them.

- Time-dependent but reversible inhibition.
- Attenuated by albumin, guanidinium, or urea.
- ⮝ the concentration of the model enzymes 10-fold largely eliminated inhibition, despite a 1000-fold excess of inhibitor
- Form particles of 30-400 nm diameter

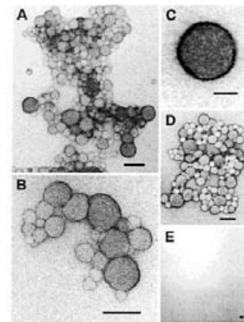
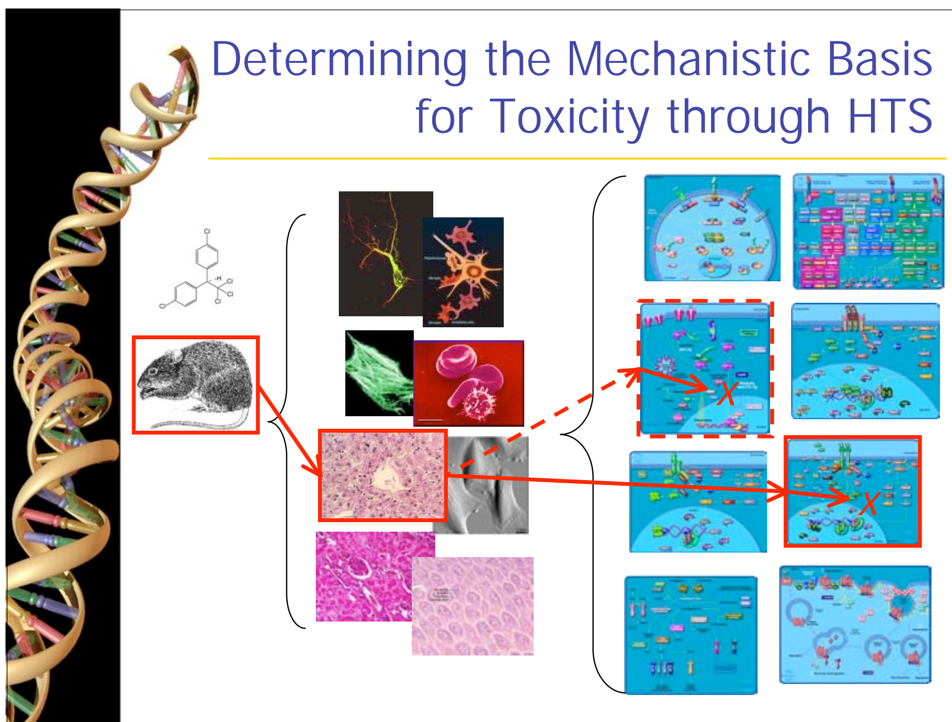


Figure 2. Compounds visualized by transmission electron microscopy: (A–C) 100 μM tetraiodophenolphthalein in 20 mM Tris; (D) 50 μM Congo Red in 20 mM Tris; (E) 625 μM ANS in 20 mM Tris. Bar = 100 nm.

Journal of Medicinal Chemistry, 2002, Vol. 45, No. 8 1717


Determining the Mechanistic Basis
for Toxicity through HTS