

Using the FLEXstation to investigate voltage-gated calcium and sodium channels

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Outline of Talk

- ◆ Ionix Profile
- ◆ Role of FLEXstation in Molecular Pharmacology Group
- ◆ Assays investigating voltage-gated ion channels:
 - ◆ Calcium
 - ◆ Sodium

Ionix Profile

- ◆ Founded July 2001, Cambridge UK
- ◆ Discovery of novel analgesic drugs
- ◆ Proprietary drug targets including ion channels
- ◆ Drug development to Phase II

Role of FLEXstation

- ◆ To play a key role in assay development
- ◆ To be used in medium throughput drug screening

- ◆ Initially used in the development of assays to investigate voltage-gated calcium and sodium channels expressed in neuroblastoma cell lines

Voltage-gated Calcium Channels

- ◆ IMR32 cells have been used to investigate calcium channels
 - ◆ Human neuroblastoma
 - ◆ Express L-type and N-type channels (differentiate for N-type)
 - ◆ Used in low throughput assays

Aim

- ◆ To develop a medium throughput fluorescence-based assay using IMR32 cells in 96-well plate format

IMR32 Cell Culture Conditions

- ◆ Cells grown in EMEM supplemented with 2mM Glutamine+1%NEAA+10%FBS
- ◆ Cells were split 1:2/1:3 every 2-3 days or had the media changed
- ◆ Differentiate with 1mM dibutyryl cAMP and 2.5 μ M bromodeoxyuridine for 8-10 days

Adherent vs Suspension Assay

- ◆ Cells used in suspension
 - ◆ IMR32 cells adhere to surfaces weakly
 - ◆ Changing media every 2-3 days causes large loss of cells when grown in 96 well plates
- ◆ Cells grown in tissue culture flasks (under differentiating conditions, if necessary) and then loaded with fluorescent indicator and assayed in suspension

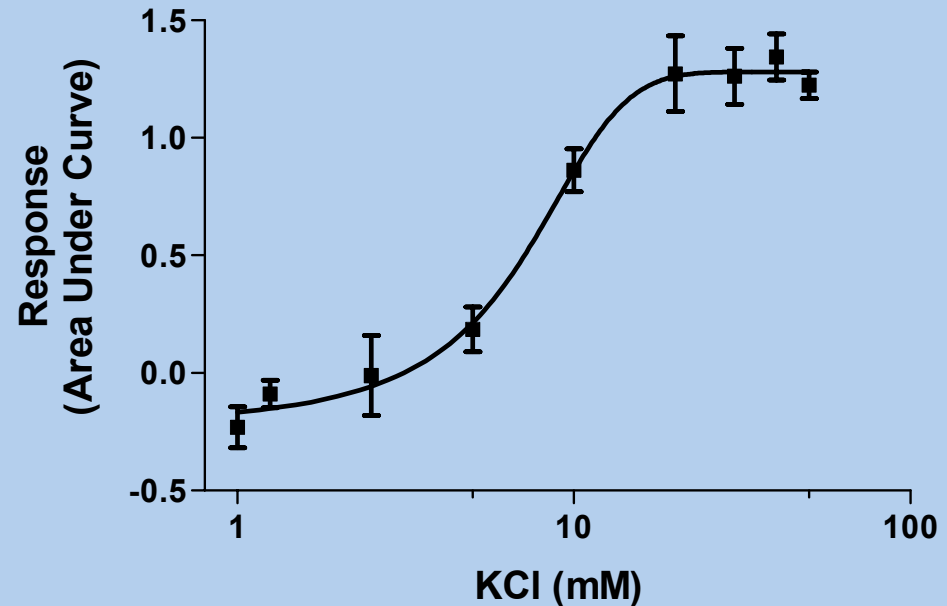
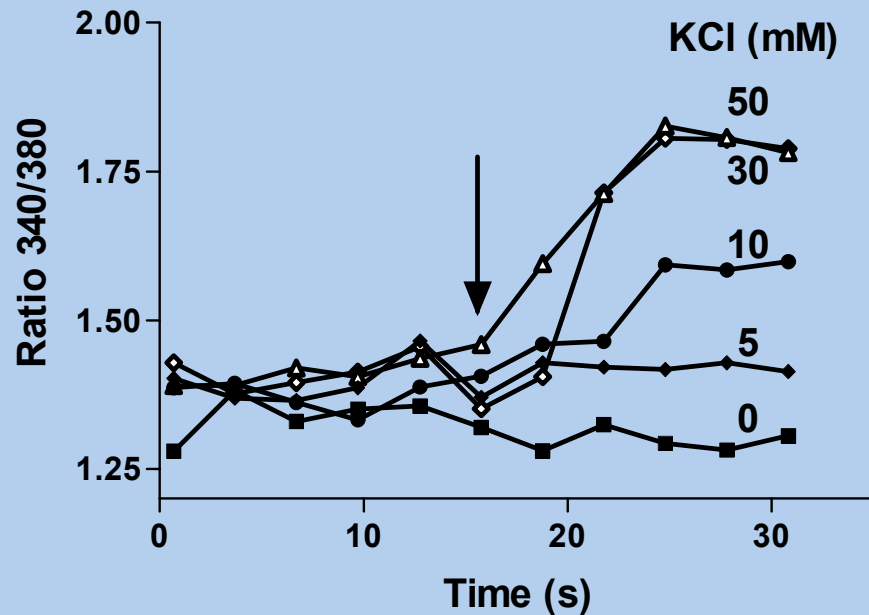
Fluorescent Indicator

- ◆ Fura-2
 - ◆ 340/380 ratio
 - ◆ Disadvantage: It cannot easily be used with a FLIPR
- ◆ Fluo-4 was used in screening assay
 - ◆ Can be used in FLIPR and hence in high-throughput assays
 - ◆ Data obtained do not require ratio calculations. Therefore are easily manipulated

Assay Protocol

- ◆ Detach cells and resuspended in assay buffer
- ◆ Incubate with 2.3 μ M Fluo-4 or 5 μ M Fura-2, 50mM probenecid for 30min with rotation
- ◆ Centrifuge and resuspend in assay buffer
- ◆ Dispense 2x10⁵ cells/well
- ◆ Assay using the FLEXstation. KCl added by fluidics system to stimulate calcium channel opening

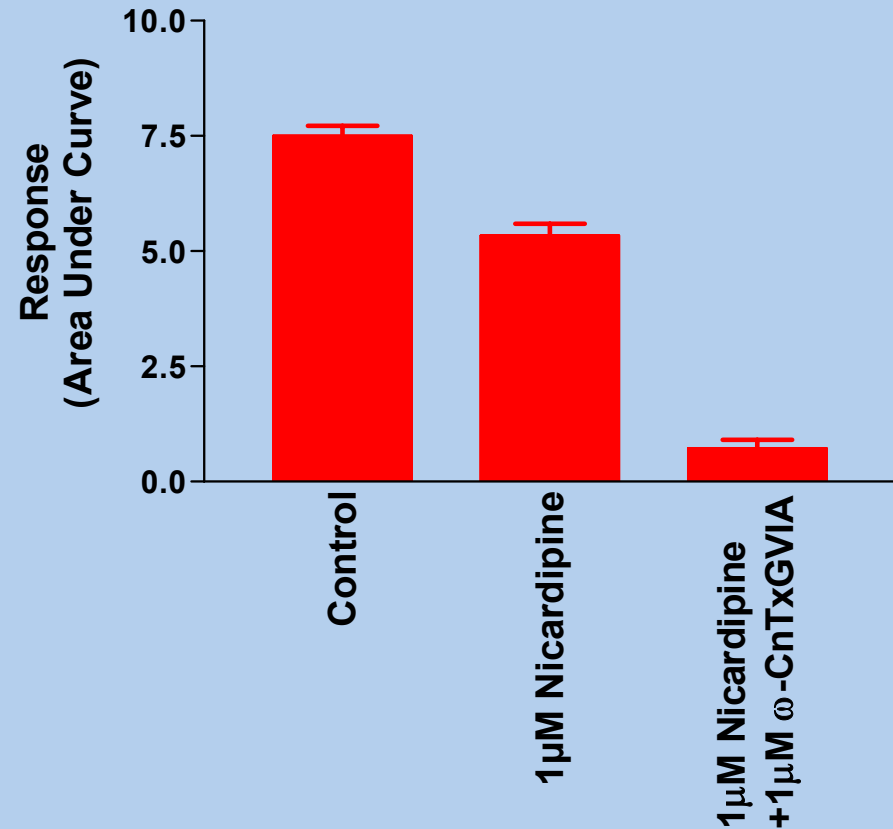
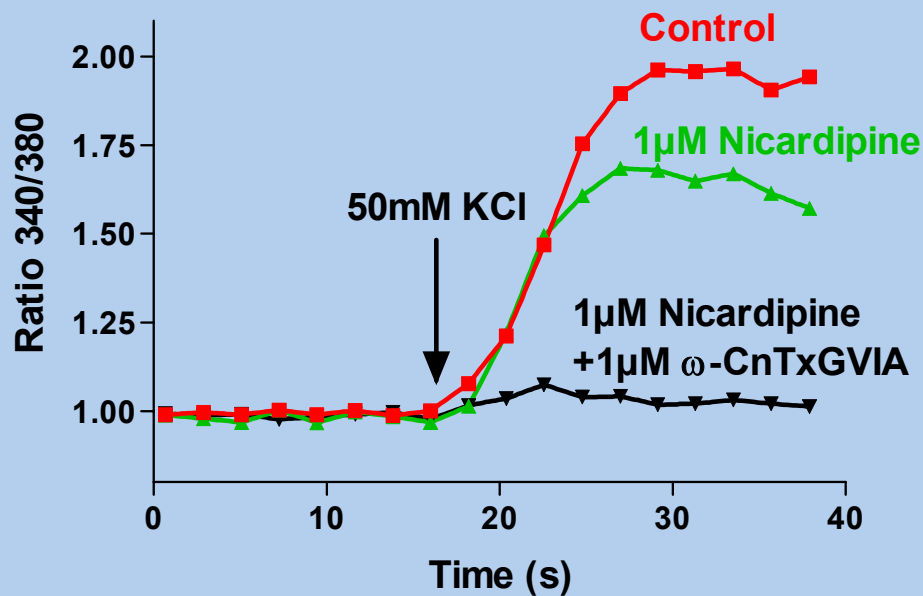
KCl-Induced Calcium Changes In IMR-32 Cells



Calcium Channel Assays

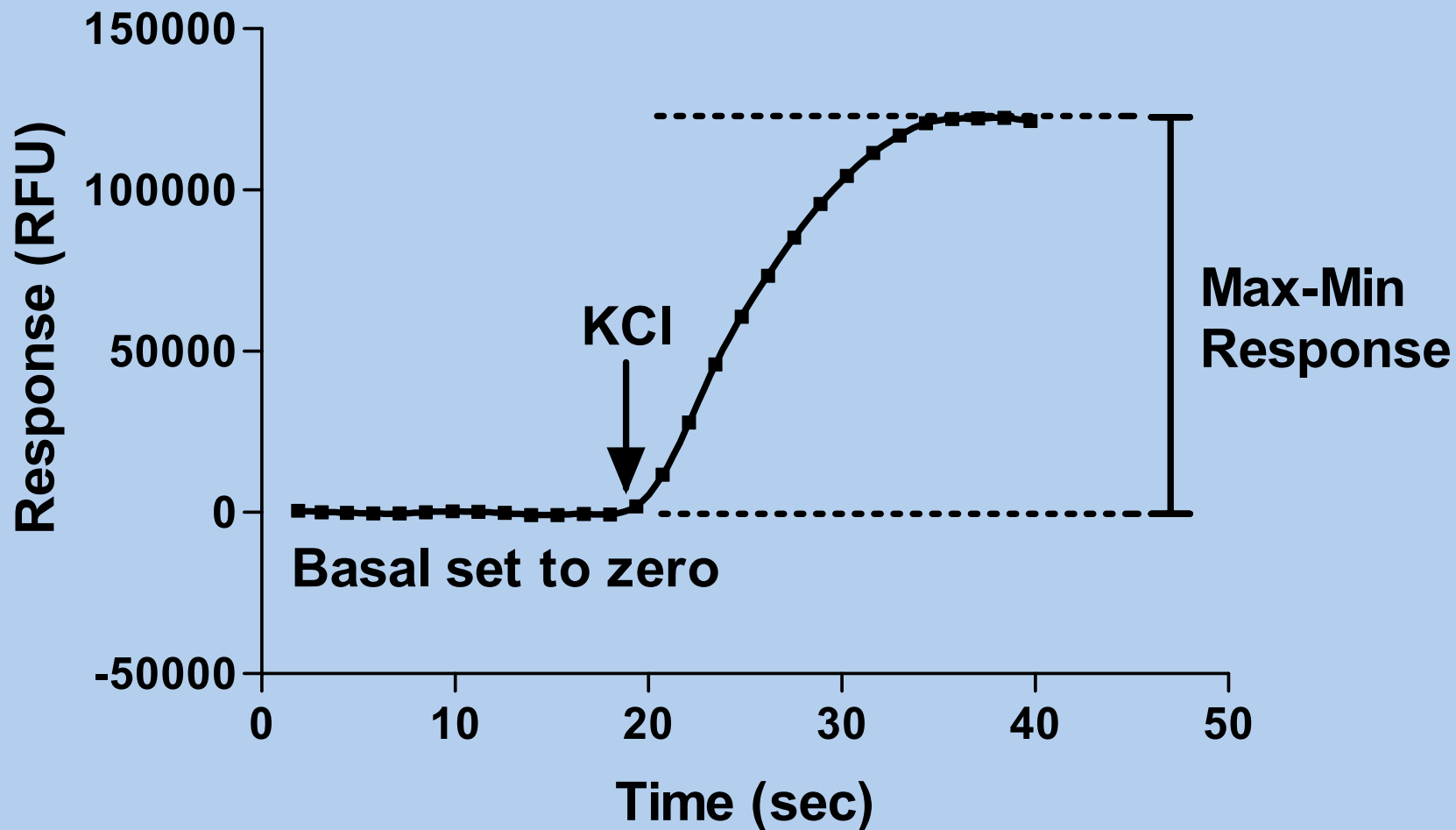
- ◆ N-Type Assay:
 - ◆ 8-10 day differentiated IMR32 cells
 - ◆ Assay run in the presence of nitrendipine or nifedipine to block L-type component
- ◆ L-Type Assay (two strategies):
 - ◆ 8-10 day differentiated IMR32 cells
 - ◆ Need to block N-type component with ω -conotoxin GVIA (expensive option)
 - ◆ Undifferentiated IMR32 cells (preferred option)
 - ◆ No N-type component

Pharmacology of Calcium Channels in Differentiated IMR-32 Cells



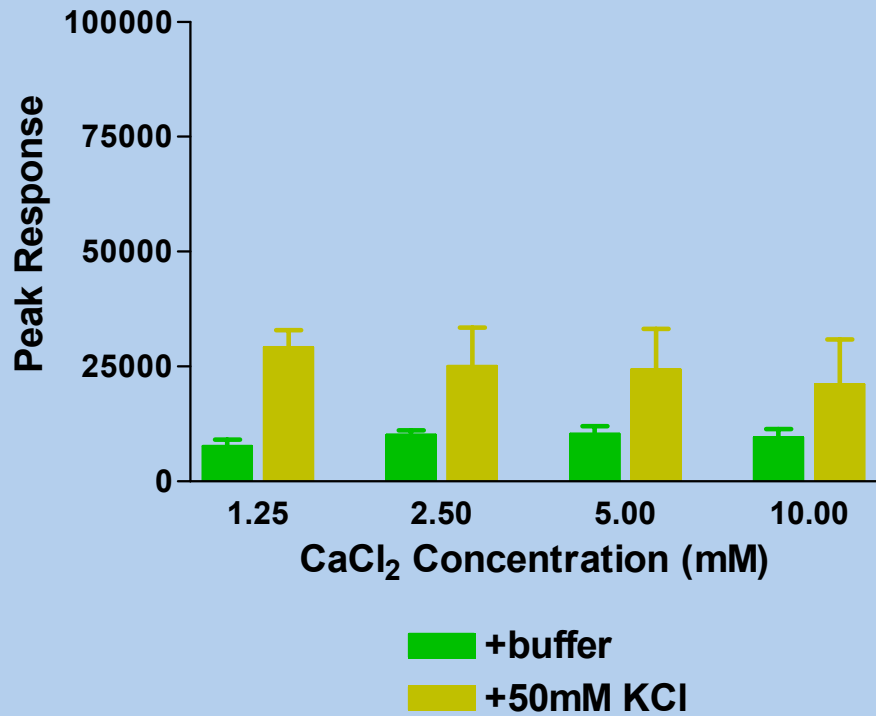
Measuring Fluo-4 Calcium Responses

FLEXstation Output

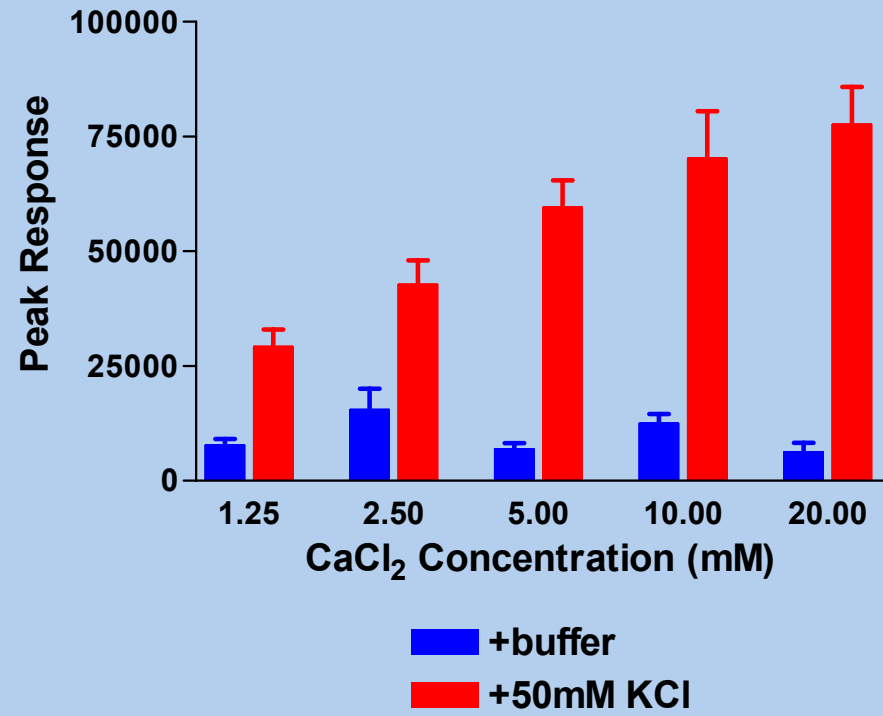


Increasing CaCl_2 Concentration in Stimulation Buffer

- ◆ Increasing CaCl_2 in assay buffer:

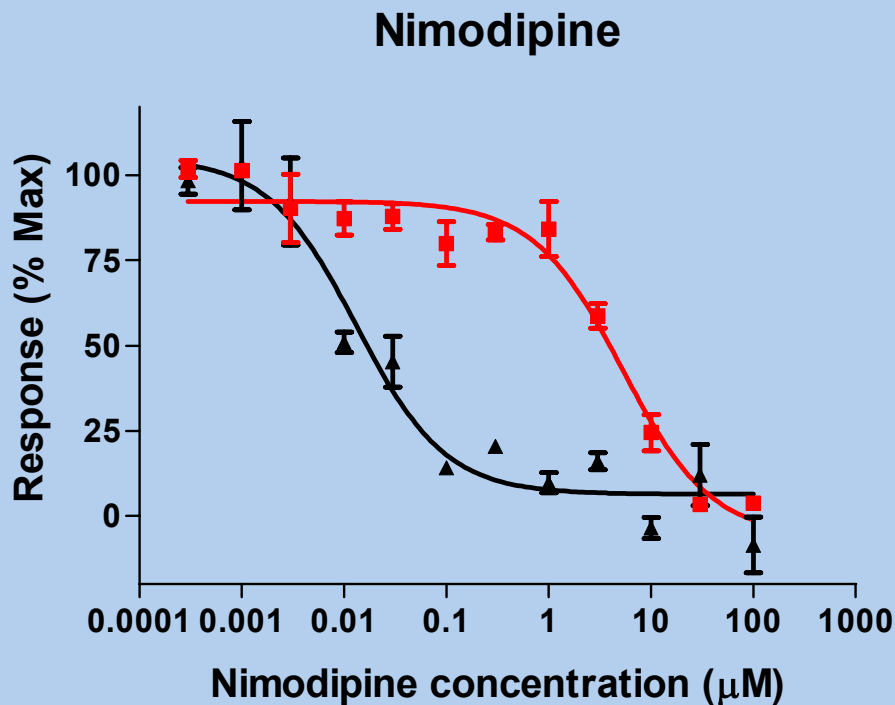


- ◆ Increasing CaCl_2 in KCl stimulation solution:

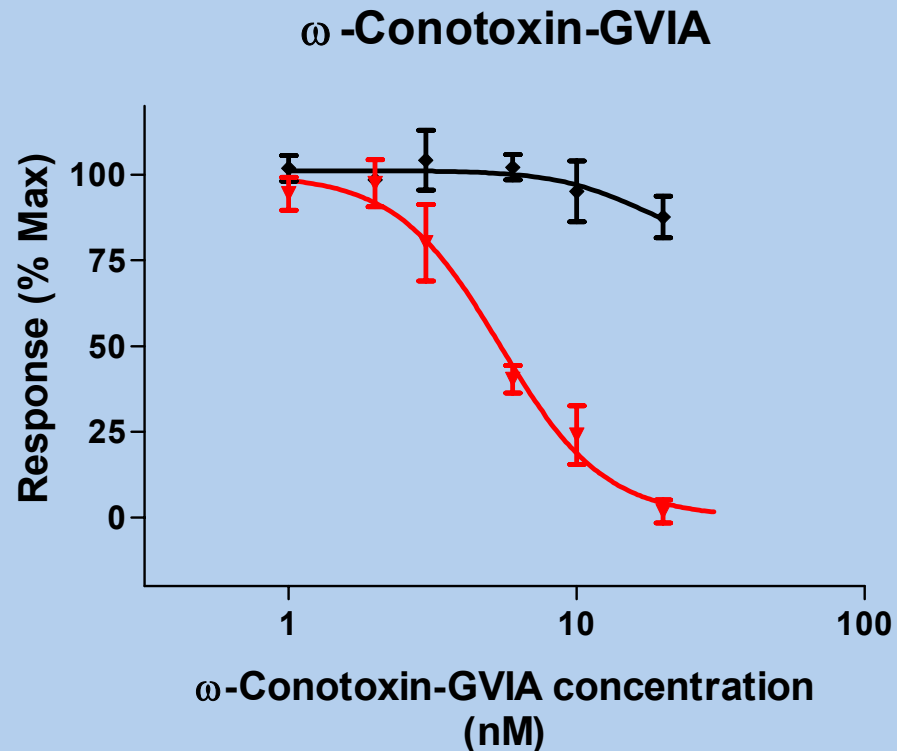


Assay Validation

- ◆ Calcium channel blockers were used to validate the assay



- N-Type IC₅₀ = 5.3 μM
- ▲ L-Type IC₅₀ = 13 nM

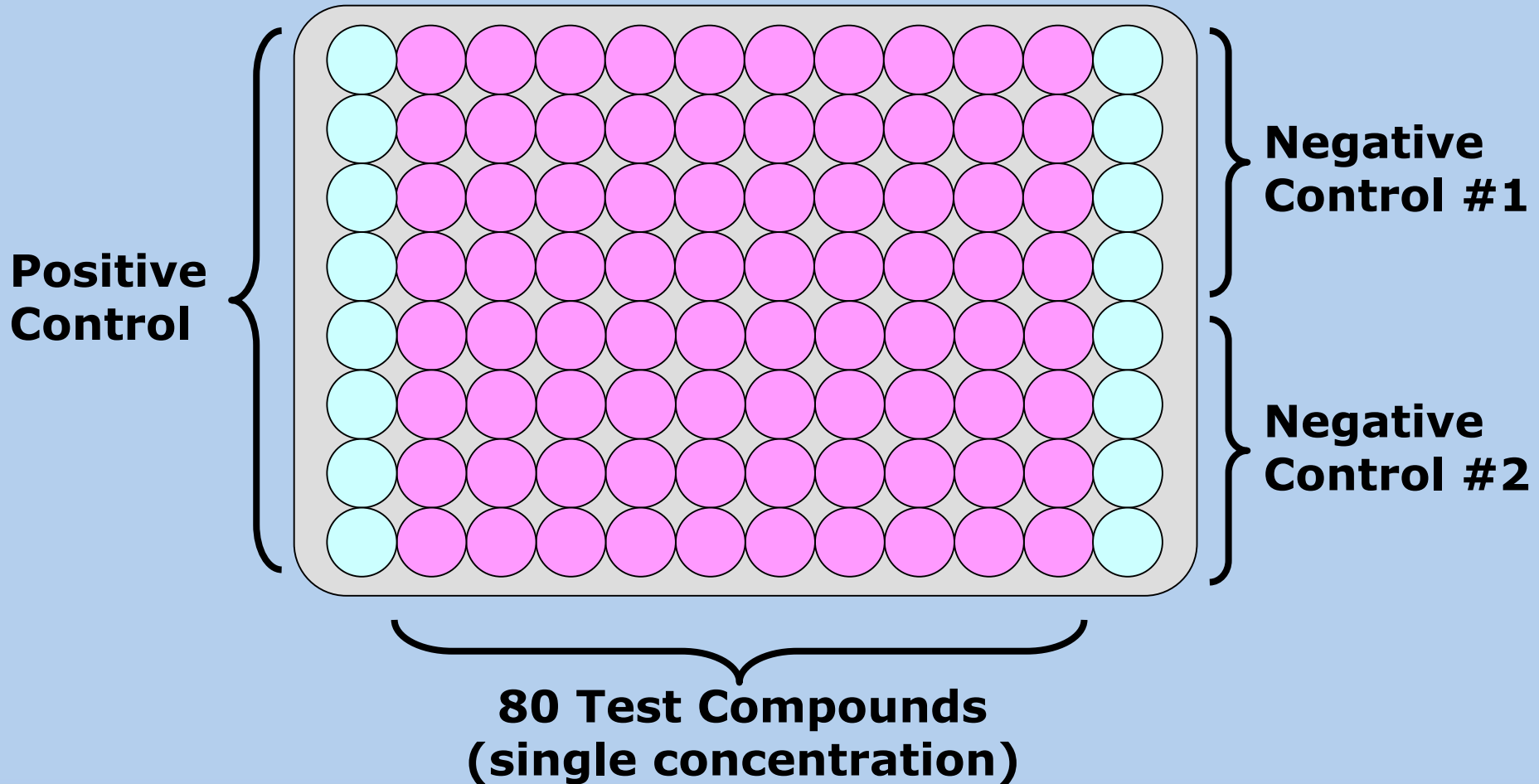


- ▼ N-type IC₅₀ = 5.5 nM
- ◆ L-type No Block

Calcium Channel Screen

- ◆ 50,000 compound library screened
- ◆ 40 plates/day on FLEXstation
- ◆ Data analyzed with SOFTmax Pro

Assay Plate Layout



SOFTmax PRO Data Analysis

SOFTmax Pro

File Edit View Experiment Control Assays Notes Window Help

No port selected Read [Icons]

Untitled

Experiment#1

Notes#1 [Icons]

Date:
Ionix ID:

Plate Validation: PENDING

Control #1: PENDING

Control #2: PENDING

Number of Hits: 0

Plate#1 [Icons]

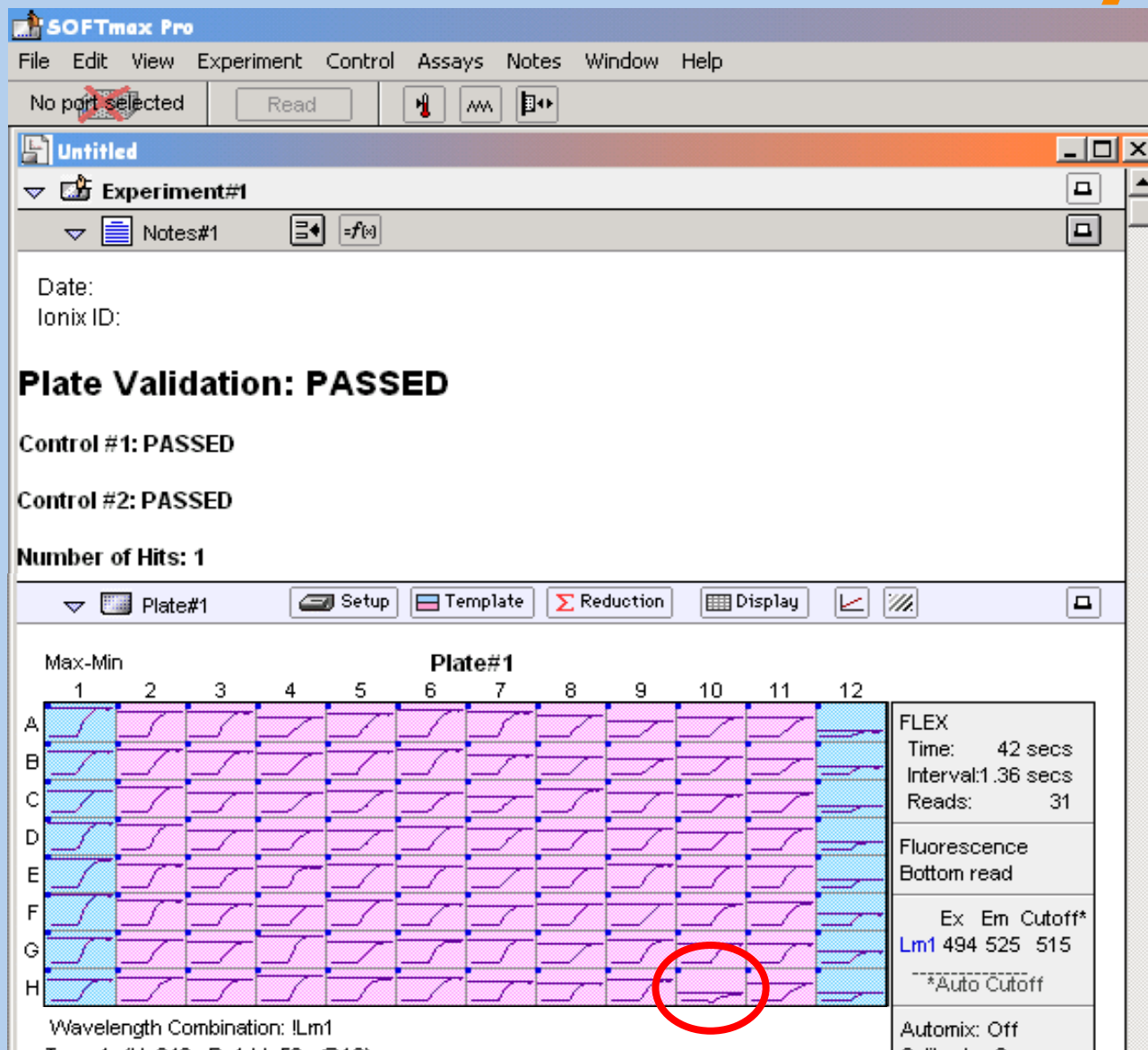
Max-Min Plate#1

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

FLEX
Time: 42 secs
Interval: 1.36 secs
Reads: 31
Fluorescence
Bottom read
Ex Em Cutoff*
Lm1 494 525 515
**Auto Cutoff
Automix: Off
Calibrate: On
DMT: High

Wavelength Combination: !Lm1
Trans1: (H=240µ,R=1,V=50µ,@18)

SOFTmax PRO Data Analysis



Summary

- ◆ Developed assay to analyze voltage-gated calcium channels on FLEXstation
- ◆ Assay now in routine use in screening laboratory

Voltage-gated Sodium Channels

- ◆ Use of Molecular Devices Membrane Potential Assay Kit
- ◆ Panel of cell lines investigated

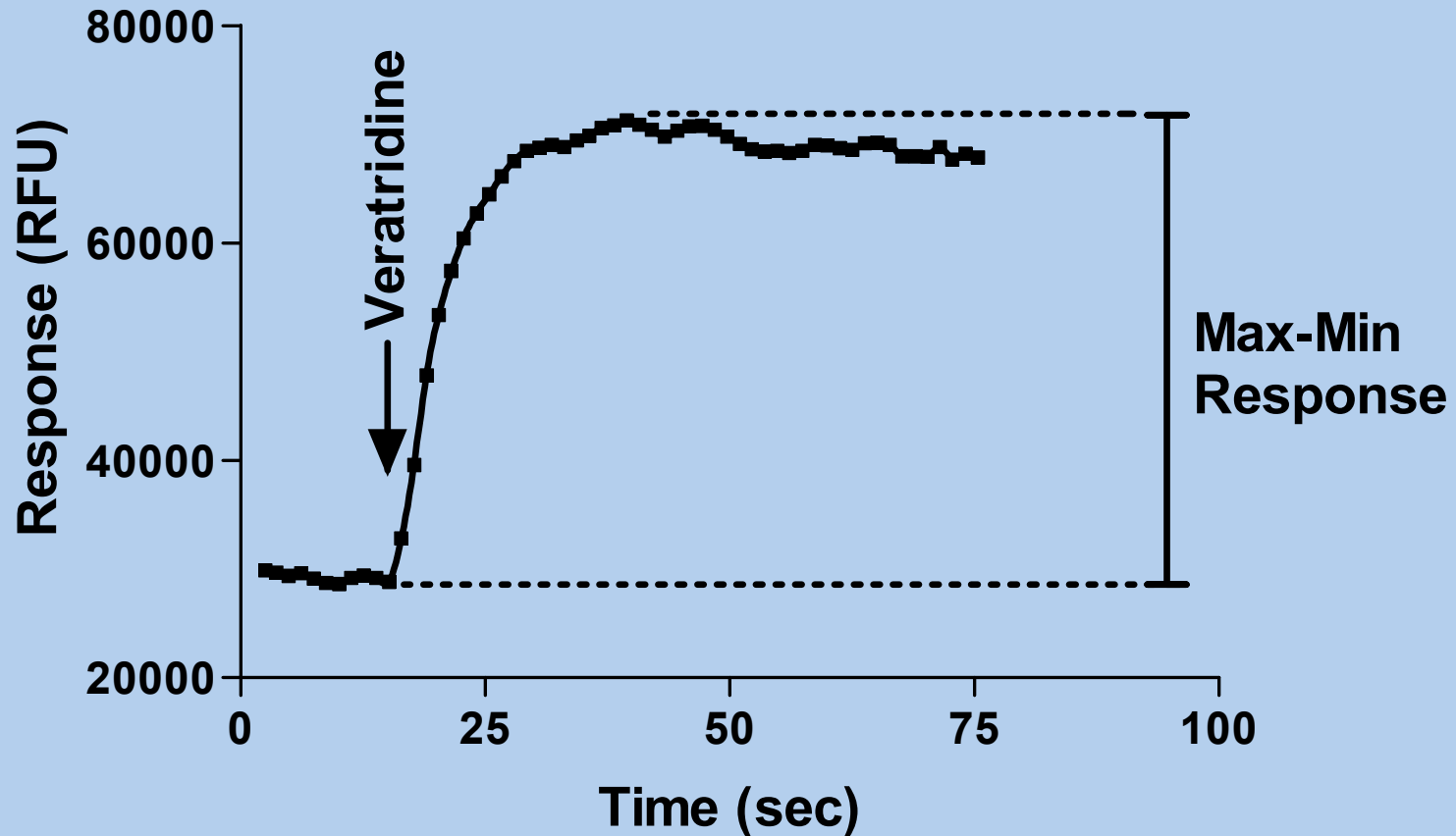
Kelly	Human neuroblastoma
BE(2)C	Human neuroblastoma
SH-SY-5Y	Human neuroblastoma
ND7/23	Hybrid of mouse neuroblastoma and rat DRG

Experimental Conditions

- ◆ Neuroblastoma cell lines grown in 96-well plates
- ◆ Dye loading according to Molecular Devices protocol
 - ◆ Except that all cells were initially loaded for 60min at 37°C
- ◆ Veratridine used to stimulate sodium channel response
 - ◆ Added by FLEXstation fluidics system

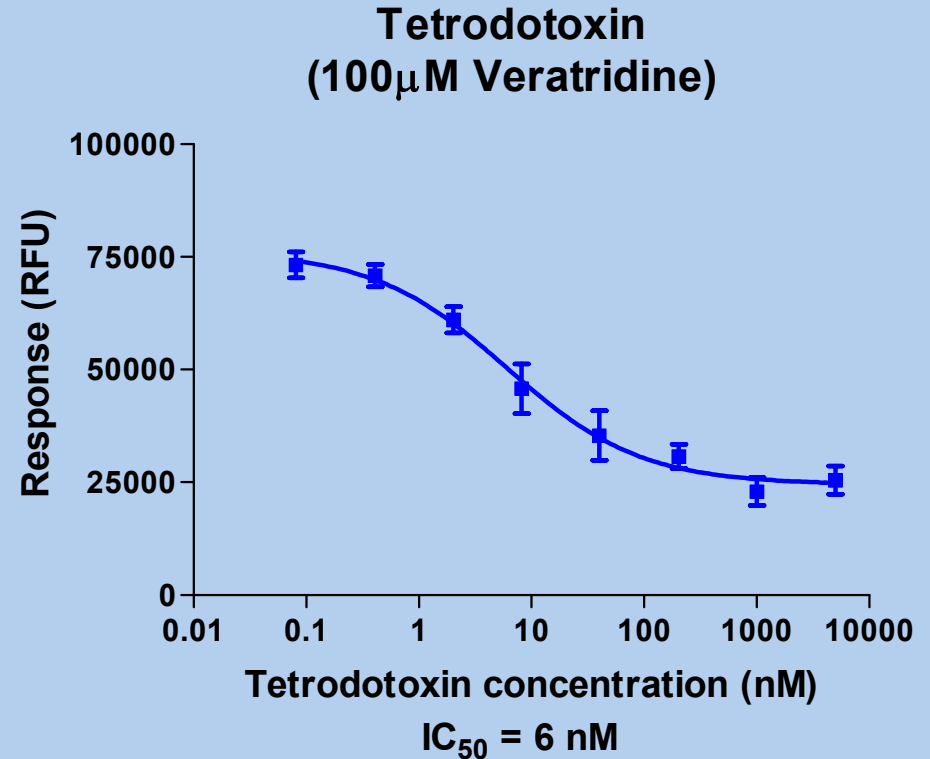
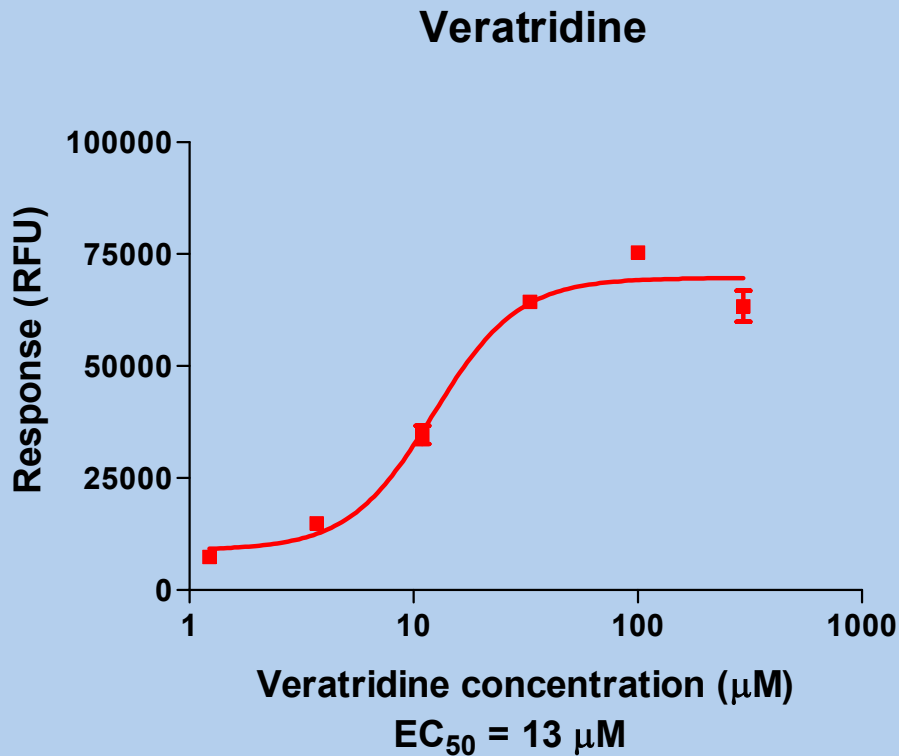
Measuring Sodium Channel Responses

FLEXstation Output



Assay Validation

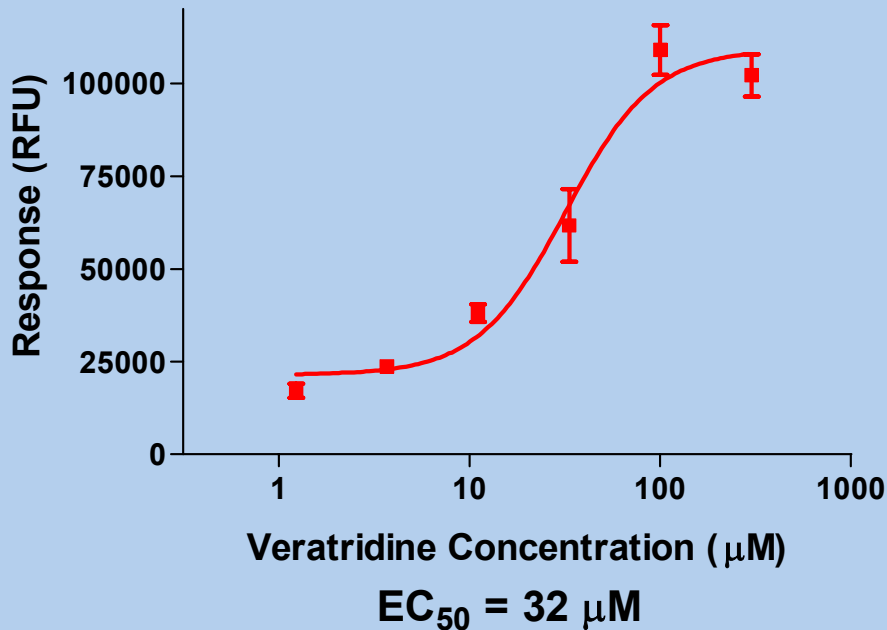
- ◆ Responses measured in BE(2)C cells



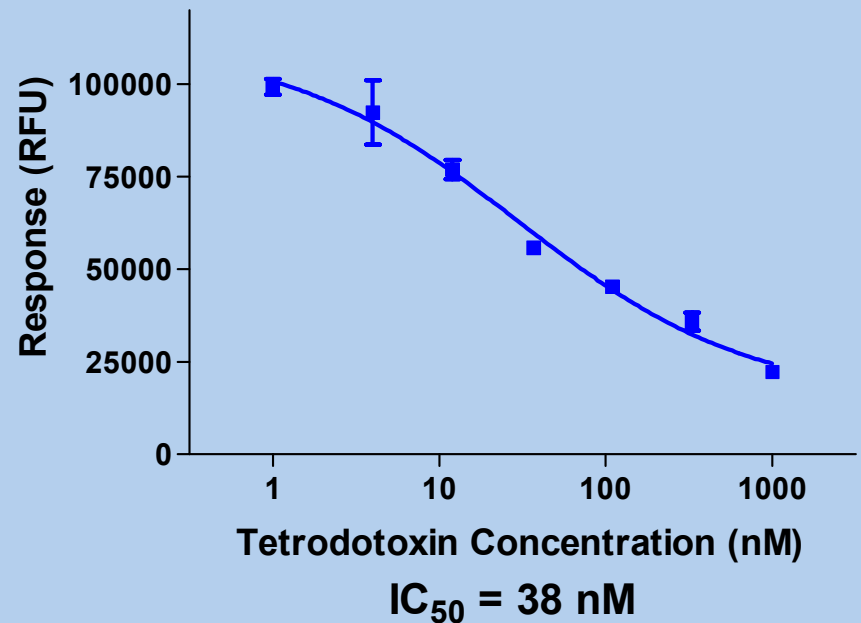
Assay Validation

- ◆ Responses measured in SH-SY-5Y cells

Veratridine



Tetrodotoxin
(100 μM Veratridine)



Summary of Data

Cell Line	EC₅₀ Veratridine	IC₅₀ Tetrodotoxin
SH-SY-5Y	24 μ M; n=6	30nM; n=3
ND7/23	29 μ M; n=6	11nM; n=3
Kelly	31 μ M; n=6	23nM; n=3
BE(2)C	24 μ M; n=6	34nM; n=3

Assay Optimisation

- ◆ Dye loading at 25°C for 30min is better than at 37°C for 60min
 - ◆ Assay carried out at 25°C
- ◆ Dye concentration can be reduced by 5 fold

Summary

- ◆ Tetrodotoxin sensitive voltage gated sodium channels have been assayed in neuroblastoma cell lines using the Molecular Devices Membrane Potential Assay Kit

Conclusions

- ◆ FLEXstation has been successfully used to...
 - ◆ Develop assays that analyze voltage-gated calcium and sodium channels expressed in neuroblastoma cell lines
 - ◆ Carry-out a medium throughput screen on voltage-gated calcium channels

Acknowledgements

- ◆ Molecular Pharmacology Group
 - ◆ Dave Thomas
 - ◆ Stuart Ward
 - ◆ Kathryn Elsegood
 - ◆ Zoe Gladwell