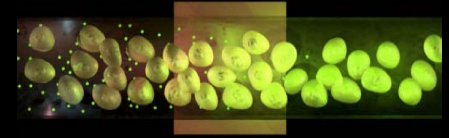


Expression of Functional Voltage-Gated Calcium & Potassium Ion Channels for Streamlined High Throughput Screening: Automated Electrophysiology Case Studies

James Brady, Ph.D.

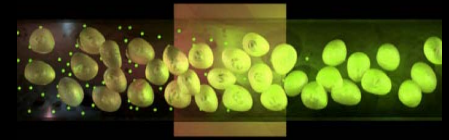
Director of Technical Applications, MaxCyte

Agenda



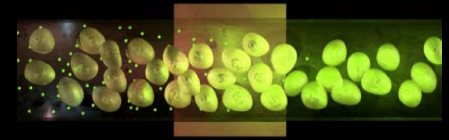
- Ion Channel Assay Challenges
- Overview of Scalable Electroporation Capabilities
- Voltage-Gated Calcium Channels
 - $Ca_v1.2$, $Ca_v2.1$, $Ca_v2.2$ & $Ca_v3.2$
 - FLIPR[®]
- Voltage-Gated Potassium Channels
 - Expression and Optimization for $K_v1.3$ & $K_v1.5$
 - IonWorks[®]
 - PatchXpress[®]

Challenges to Creating Cell-Based Assays for Ion Channel Screening



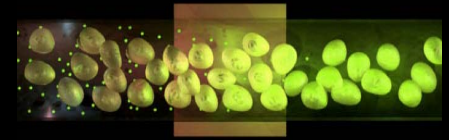
- Construction and validation of stable cell lines is very labor-, time- and cost-intensive.
- Many functional ion channels are heteromers of multiple pore-forming and modulatory subunits.
- Co-expression of multiple subunits necessitates multiple antibiotic selection markers resulting in unhealthy and slow-growing cells.
- Choice of inducible or constitutive expression is dependent upon ion channel type; expression can be lost over time in some instances.

Creating Cell Based Assays via Transient Transfection



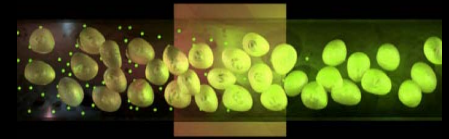
- Transient transfection is preferred over stable transfection
 - Decreased time and cost of production
 - Control over expression
 - Ability to express toxic gene products
- Multiple options for transient transfection
 - Chemical methods (e.g., lipids, CaPO_4 , PEI)
 - Viral vectors
 - Electroporation
- Limitations to above methods have prevented widespread adoption for assay development

Ideal Attributes of a Transient Transfection System for Cell Based Assay Development



- | | |
|--|---|
| <ul style="list-style-type: none">• Works with multiple cell types• High efficiency & high viability• Reproducible from day to day and scientist to scientist• User friendly• Loads multiple types of molecules & combinations of molecules• Compatible with a variety of assay formats | <ul style="list-style-type: none">• Allows cryopreservation of transfected cells• Suitable for multiple classes of targets• Scalable• Process compatible• Short assay development time• Cost effective |
|--|---|

MaxCyte® STX™ Scalable Transfection System

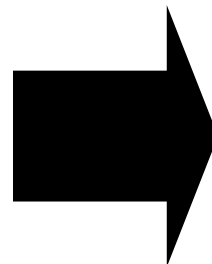


Proprietary Electroporation Technology



- Simple:** Fast and easy
- High Yield:** >90% viability
- High Efficiency:** >90% transfection efficiency
- Safe:** Chemically defined buffer
No added biological agents
Sterile, closed system
- Scalable & Rapid:** 5×10^5 - 4×10^7 (**Static EP**) in seconds
 1×10^{10} cells (**Flow EP**) in <30 min
- Rugged:** Reproducible & consistent
- Quality:** cGMP compliant
ISO 9001 certification
CE Marking

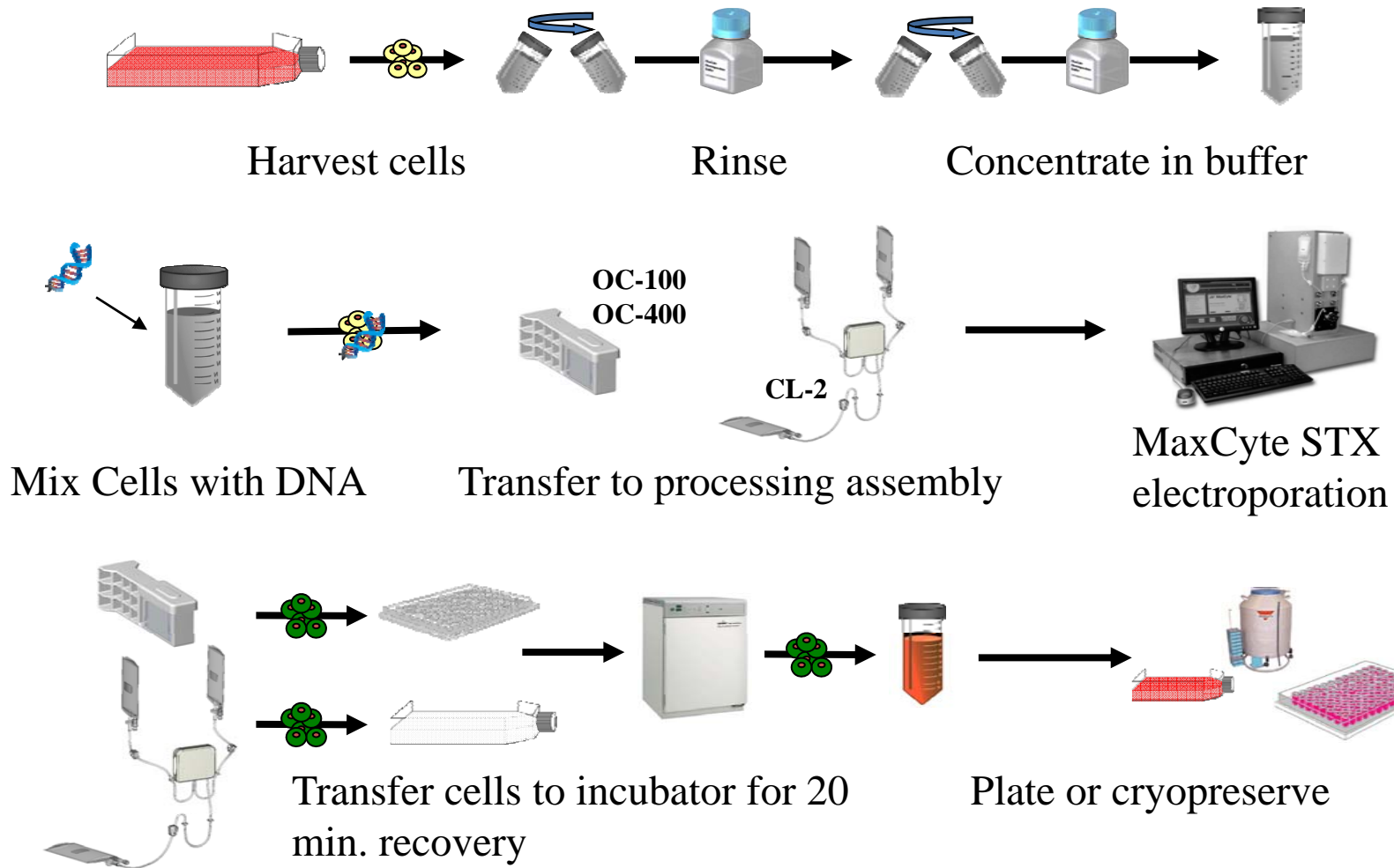
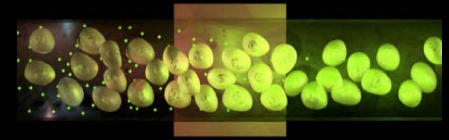
Small molecules
Antigens (proteins/lysates)
Nucleic acids (DNA, mRNA, siRNA)



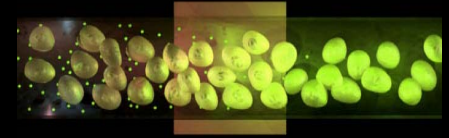
Primary cells
Stem cells
Mammalian cell lines



MaxCyte STX Electroporation Process

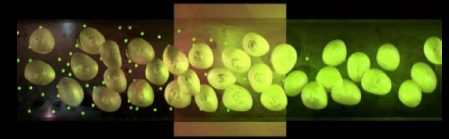


Keys to Successful Assay Development with the MaxCyte STX System

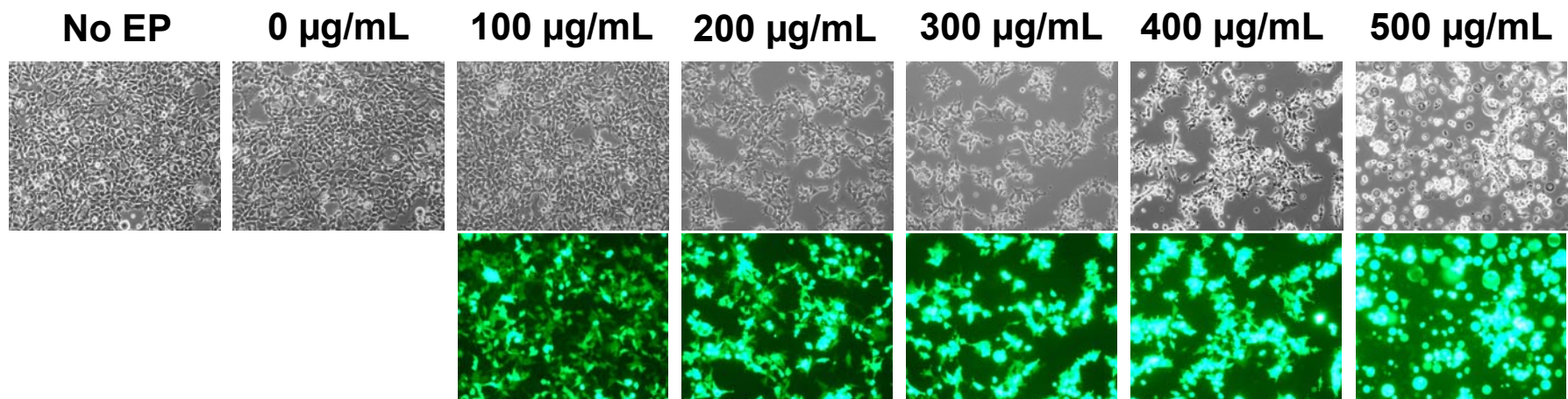
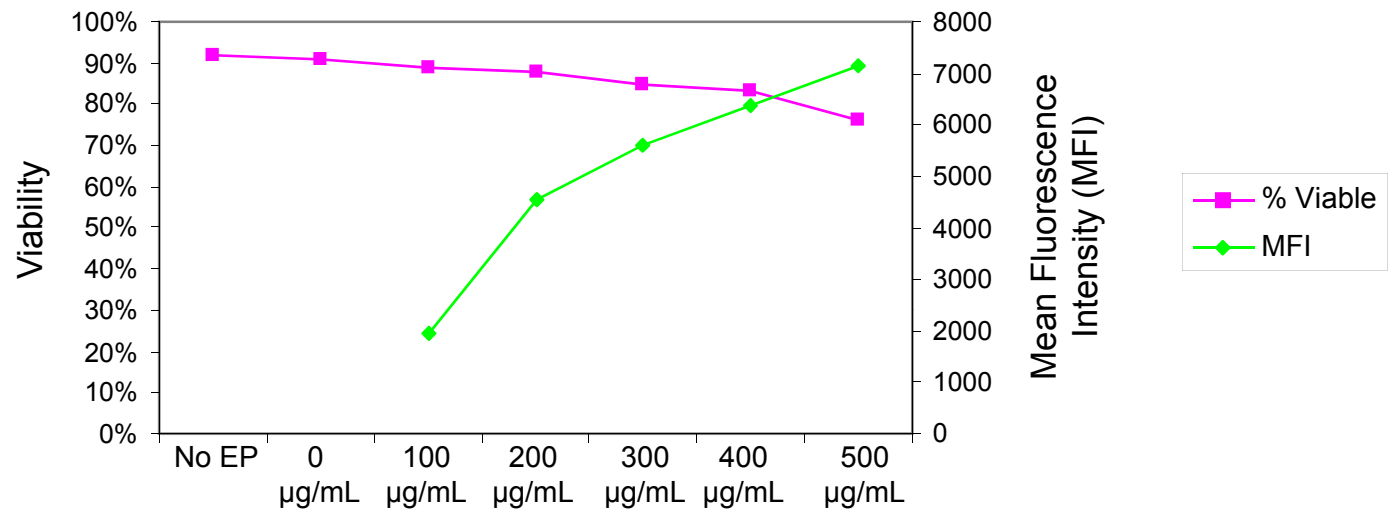


1. DNA Preparation
2. Cell Culture
3. Electroporation and DNA Titration
4. Cell Handling Post-EP
5. Plating and Analysis
6. Scale-Up
7. Cryopreservation

Optimizing DNA Concentration for Transfecting HEK 293H Cells



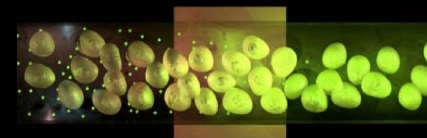
24 hrs
post EP



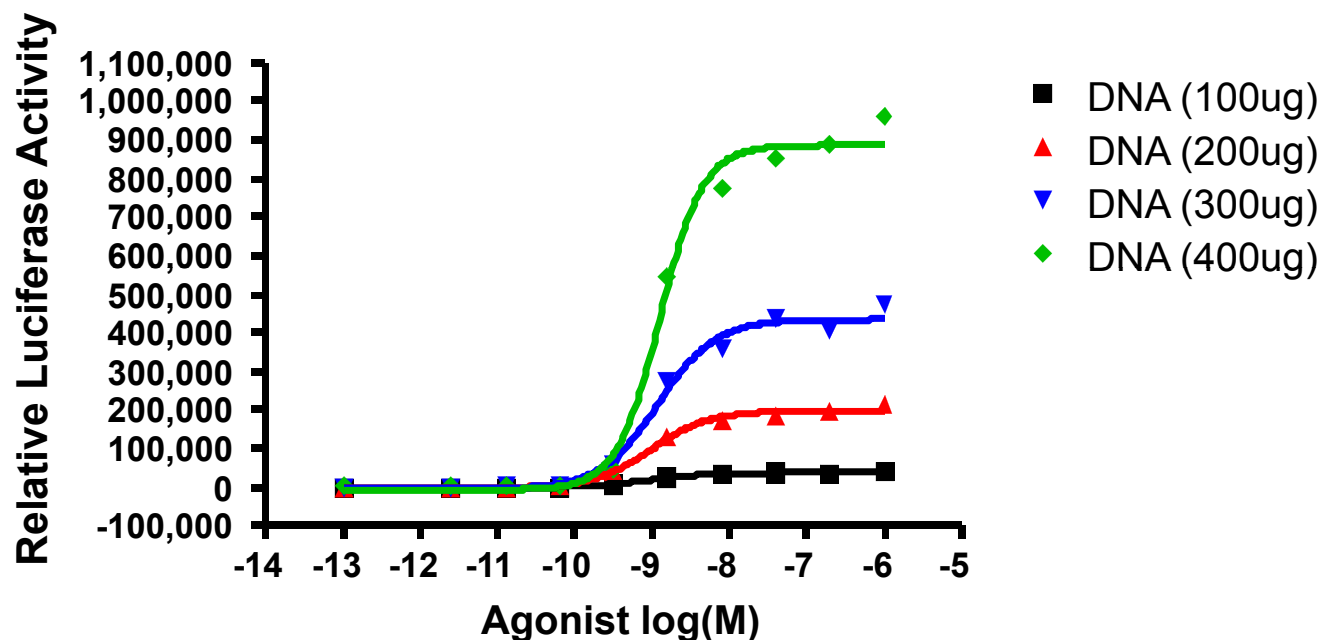
98-100% GFP+



Nuclear Receptor Assay Optimization in Transiently Transfected U2OS Cells

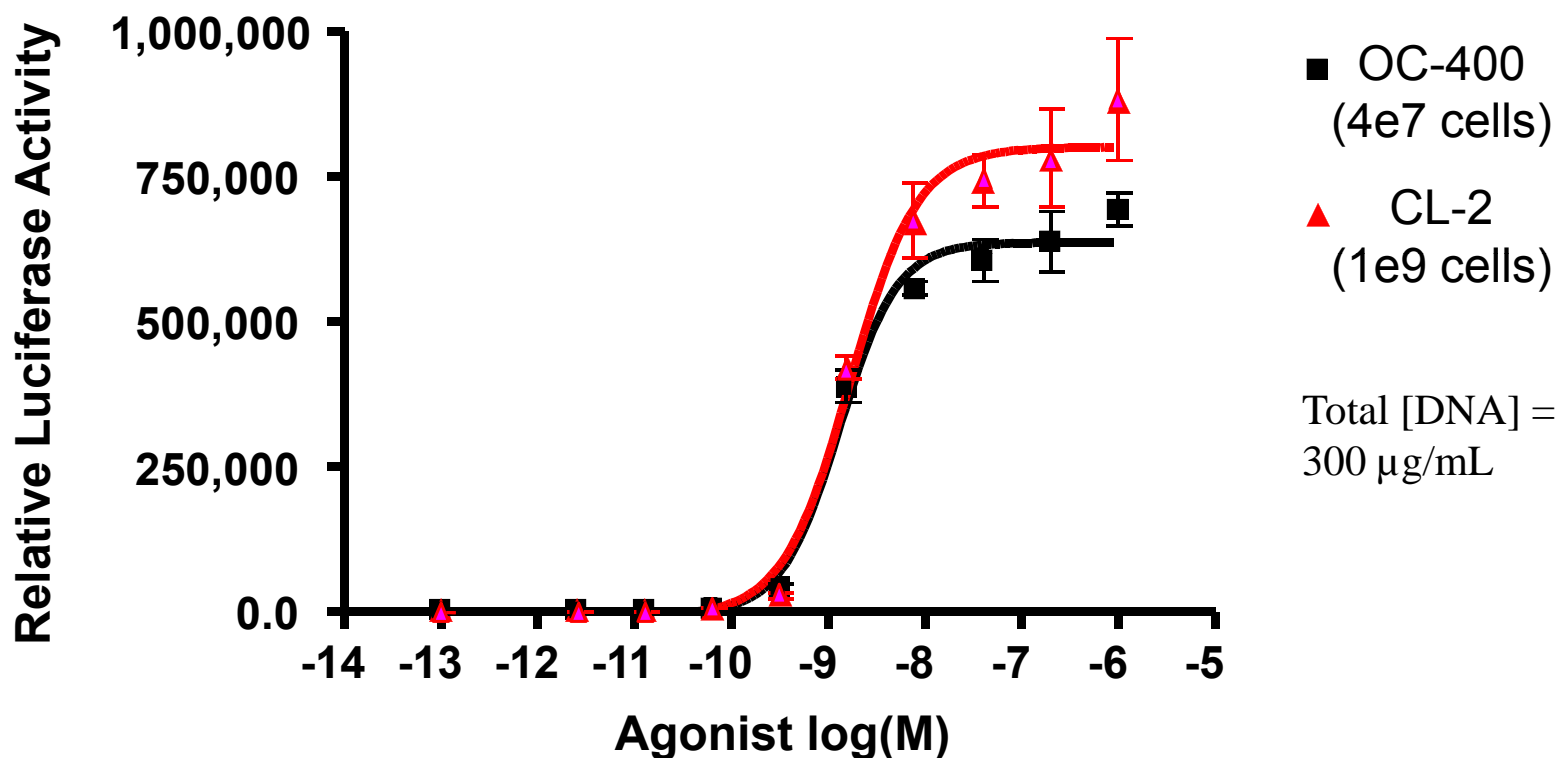
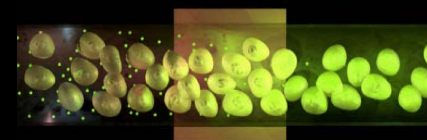


- U2OS cells co-transfected with equimolar ratio of NR & reporter plasmids
- Assayed 24 hrs following static electroporation in OC-400 PAs (4e7 cells/EP)



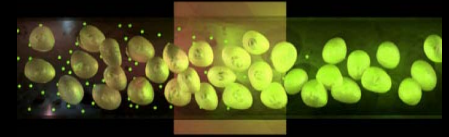
	100 µg/mL	200 µg/mL	300 µg/mL	400 µg/mL
EC50	9.33E-10	9.78E-10	1.20E-10	1.27E-10
Window	66.67	204.61	310.14	378.38

Scaling a Nuclear Receptor Assay with Transiently Transfected U2OS Cells



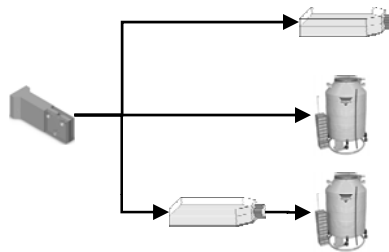
	OC-400	CL-2
EC50	1.31E-09	1.69E-09
Window	347.6	332.3

Cryopreserving Transfected Cells

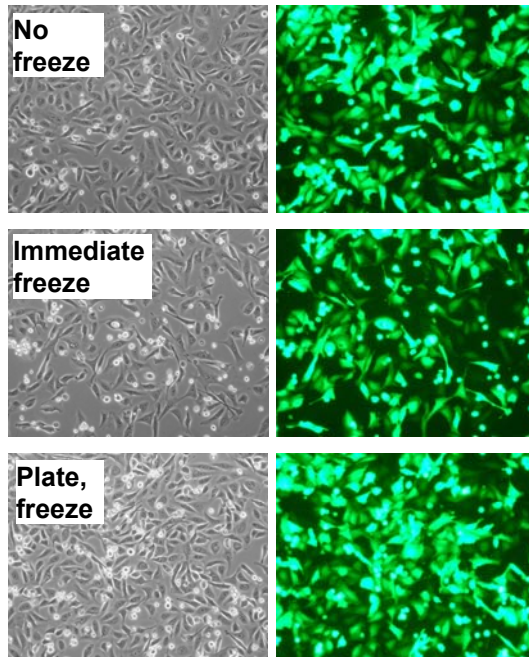


U2OS Cells + 200 µg/mL pGFP

- 1/3 Plate without freezing (**No freeze**)
- 1/3 Freeze after 20 min. post EP recovery (**Immediate freeze**)
- 1/3 Plate before freezing (**Plate, freeze**)

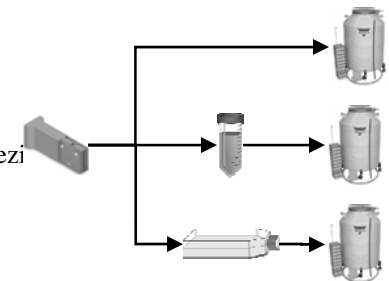


24 hrs

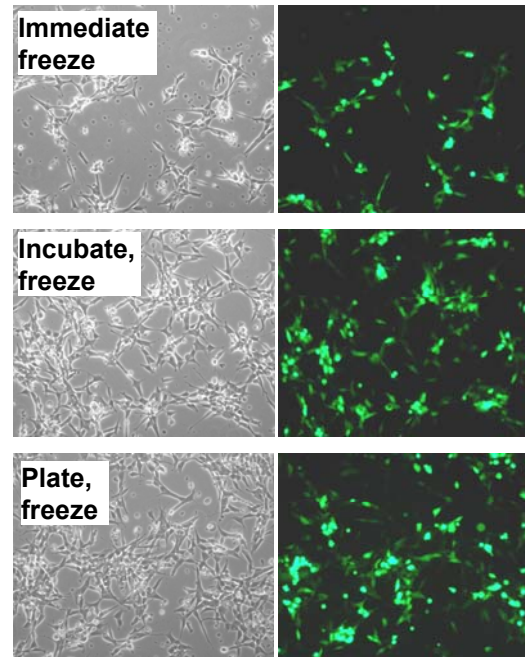


SH-SY5Y Cells + 100 µg/mL pGFP

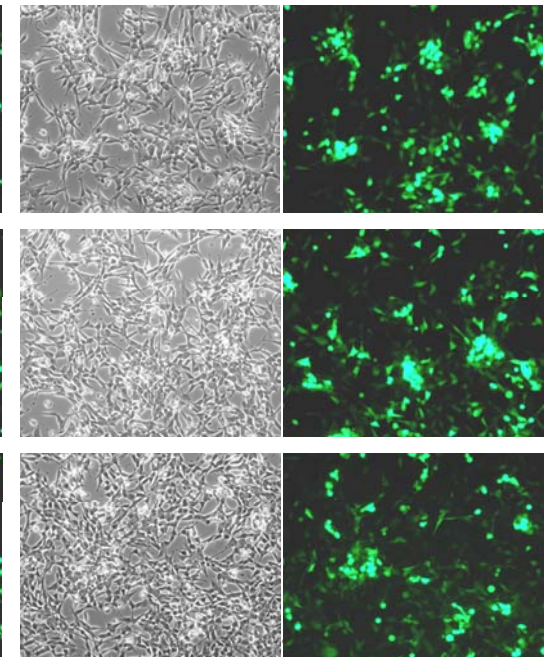
- 1/3 Freeze after 20 min. post EP recovery (**Immediate freeze**)
- 1/3 Add medium after 20 min. & incubate 1 hr @ 37°C before freezing (**Incubate, freeze**)
- 1/3 Plate before freezing (**Plate, freeze**)

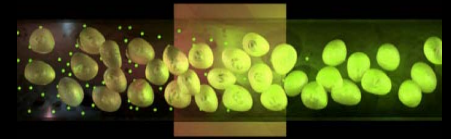


24 hrs



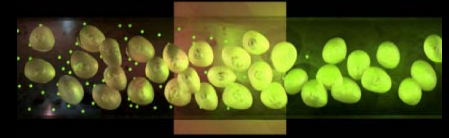
48 hrs





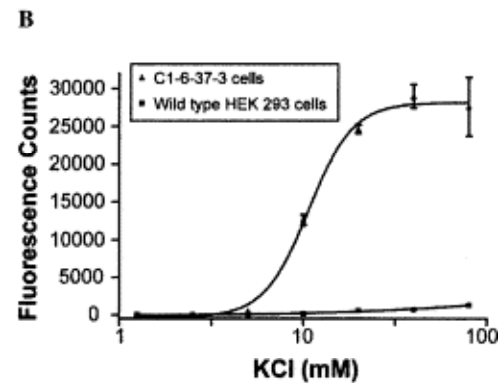
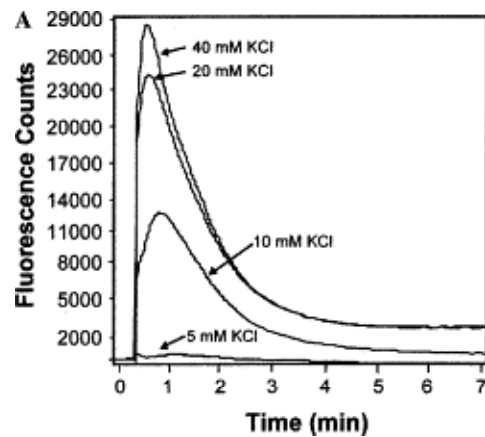
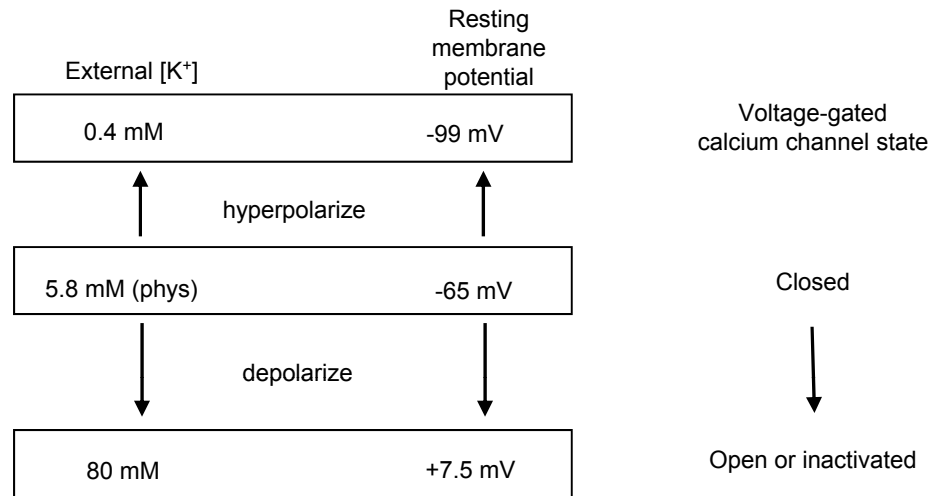
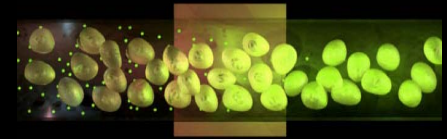
Screening Ca_v Ion Channels with the FLIPR^{Tetra}®

Voltage-gated Ca Channel (Ca_v) Transient Transfections with the MaxCyte STX System

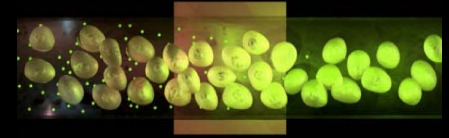


- Transfect HEK 293 cells with equimolar ratio of four ion channel cDNAs:
 - Ca_v pore-forming alpha subunit (~5-6 kb cDNA)
 - Modulatory β subunit
 - Modulatory $\alpha 2\delta$ subunit
 - Inward rectifier potassium channel (Kir2.1) to allow modulation of resting membrane potential by external K^+
- Plate in 384 well plate 20 min. post EP
- Perform FLIPR^{TETRA}[®] calcium flux assays @ 24 & 48 hrs post EP

Coexpression of an Inward Rectifier & Ca_v



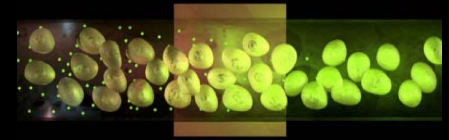
FLIPR^{TETRA}® Assay for Ca_v Activity



Calcium influx assay was performed with the Calcium-4 No Wash Kit (MDS-AT):

1. Dye loading for 30 minutes
2. Antagonist or vehicle was added for an additional 30 minute incubation
3. Cells were depolarized with high external K⁺ (up to 135 mM)
4. Signal to noise ratios for individual Ca_v types were optimized by varying external [K⁺] and [Ca²⁺]

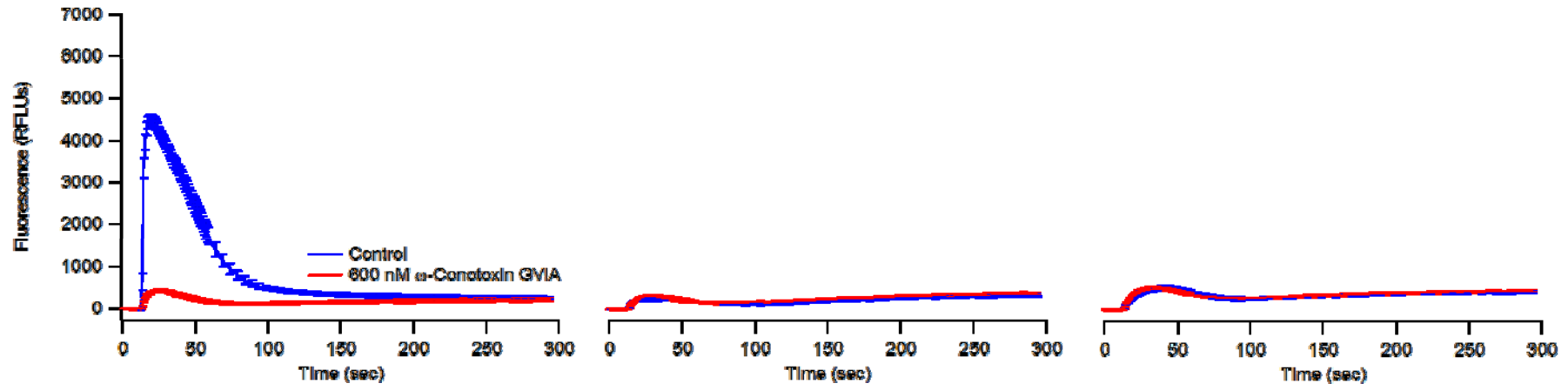
Calcium influx in HEK 293 cells transfected with $Ca_v2.2$, $\beta3$, $\alpha2\delta$ & Kir2.1



Cav2.2 + $\beta3$ + $\alpha2\delta$ + Kir2.1

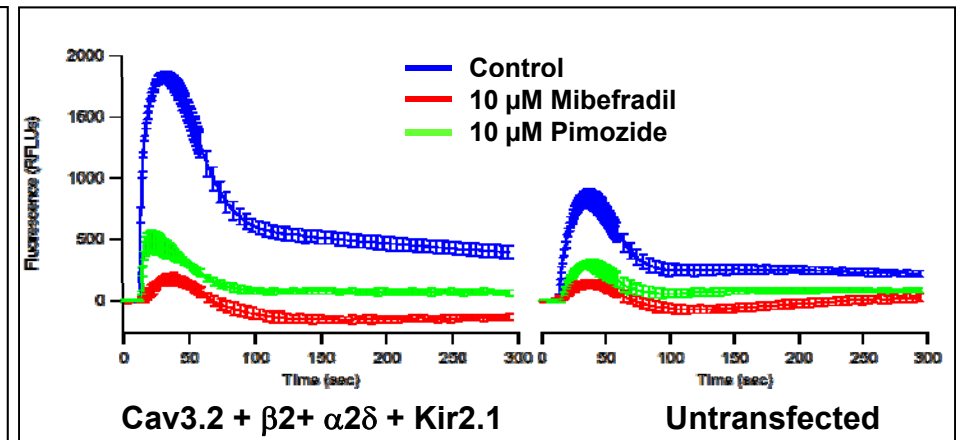
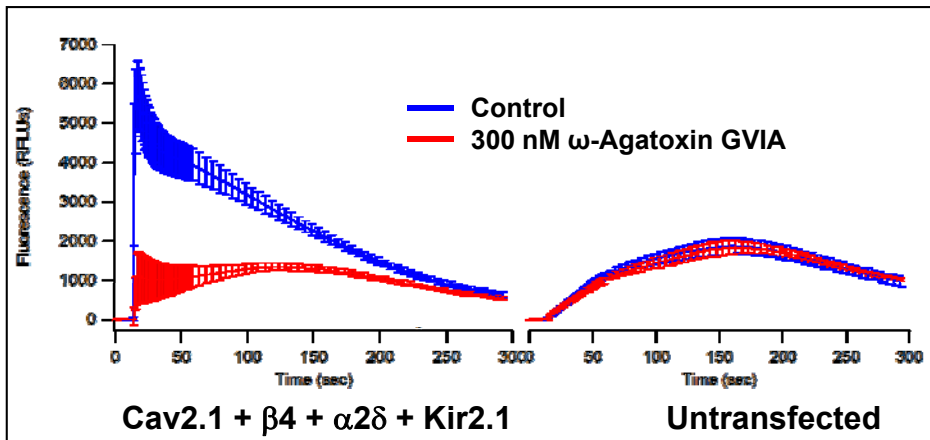
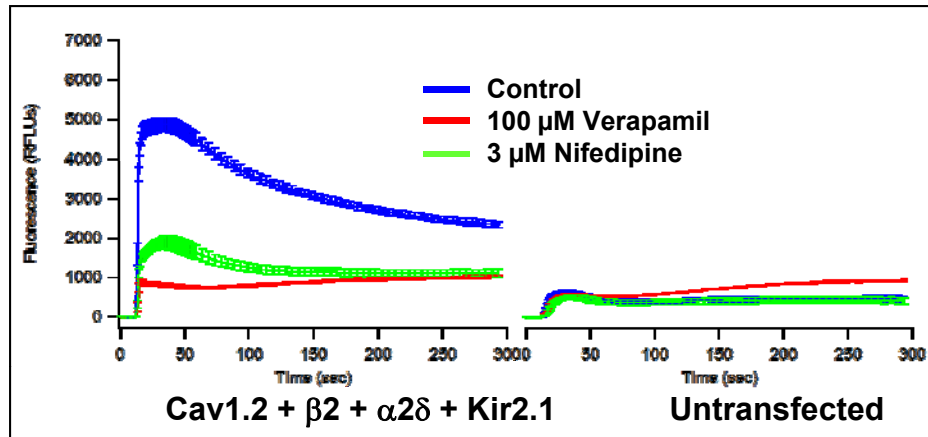
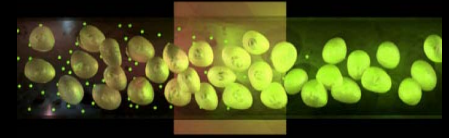
Cav2.2 + $\beta3$ + $\alpha2\delta$

Untransfected

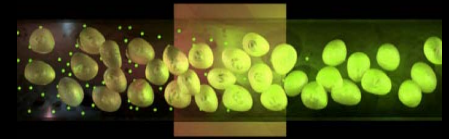


Controls: n = 8; antagonist groups: n = 4; Error bars = Std. Dev.

Ca Influx Measured with FLIPR^{TETRA}® 24-48 hrs Post Transfection

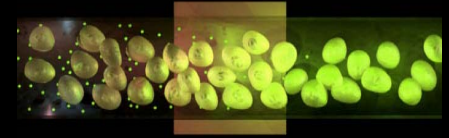


Controls: n = 8; antagonist groups: n = 4; Error bars = Std. Dev.



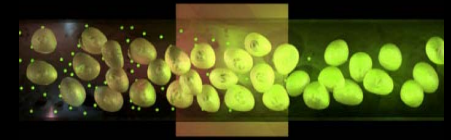
Screening K_v Ion Channels with the IonWorks® Quattro™ and the PatchXpress® 7000A

Transfecting CHO K1 Cells with $K_v1.5$ α -Subunit Plasmid DNA



- Static electroporation in OC-400 PAs
- Incubation at 37°C for 24 hrs followed by incubation at 28°C for 24 hrs
- Analysis on IonWorks[®] Quattro[™]

K_v Ion Channels : Good Seal Performance & Strong Functional Expression

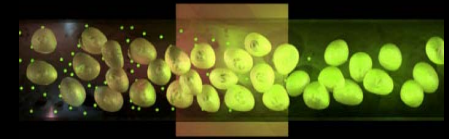


Condition	% seals (>100 MΩ)	Seal resistance (mean±SD)	% expression (>0.5 nA)	Current amplitude (mean±SD)
Non-transfected	97%	322±136 MΩ	-	-
50 µg/ml cDNA	87%	295±101 MΩ	54%	1.8±1.5 nA
100 µg/ml cDNA	84%	278±83 MΩ	89%	2.9±1.8 nA
150 µg/ml cDNA	96%	246±78 MΩ	95%	3.2±2.1 nA

High seal efficiency & resistance

Gene expression correlates with DNA concentration

K_v Ion Channels : MaxCyte STX vs. Lipid Based Transfection

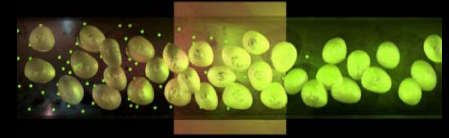


Condition	PatchPlate	% seals (>100 MΩ) ¹	Seal resistance (mean±SD)	% expression (>0.5 nA)	Current amplitude (mean±SD)
Lipid-Mediated Transfection (20 ug DNA + 60 ul commercial lipid reagent)	Single Hole (SH)	77%	191±46 MΩ	4%	1.1±1.0 nA
150 µg/ml cDNA 48 hrs post-transfection	Single Hole (SH)	82%	248±87 MΩ	93%	2.8±1.4 nA
	Population Patch Clamp (PPC)	100%	72±31 MΩ	98%	1.3±0.3 nA

¹PPC seals >20 MΩ

Data obtained from IonWorks® Quattro™

Large Scale Transfection & Cryopreservation of Cells for Ion Channel Screening



- 8e8 CHO K1 cells transfected with 150 µg/mL K_v1.5 plasmid DNA using flow electroporation
- Cultured @ 37°C for 24 hrs and @ 28°C for additional 24 hrs
- Cryopreserved @ 2e6 cells/vial

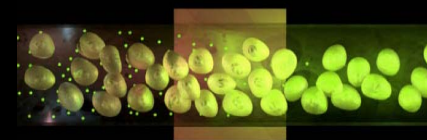
Date	PatchPlate	% seals (>100 MΩ) ¹	Seal resistance (mean±SD)	% expression (>0.5 nA)	Current amplitude (mean±SD)
August 2009	SH	50%	202±77 MΩ	86%	2.7±1.3 nA
	PPC	98%	38±11 MΩ	99%	1.5±0.3 nA
January 2010	SH	53%	197±80 MΩ	81%	2.4±1.2 nA
	PPC	93%	30±8 MΩ	97%	1.4±0.3 nA
	PPC	90%	32±8 MΩ	100%	1.9±0.2 nA
	PPC	99%	34±8 MΩ	100%	1.4±0.2 nA

¹PPC seals >20 MΩ.

Data obtained from IonWorks® Quattro™

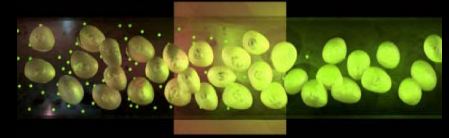
K_v Ion Channels : Cryopreserved Cells

K_v1.5 Pharmacology

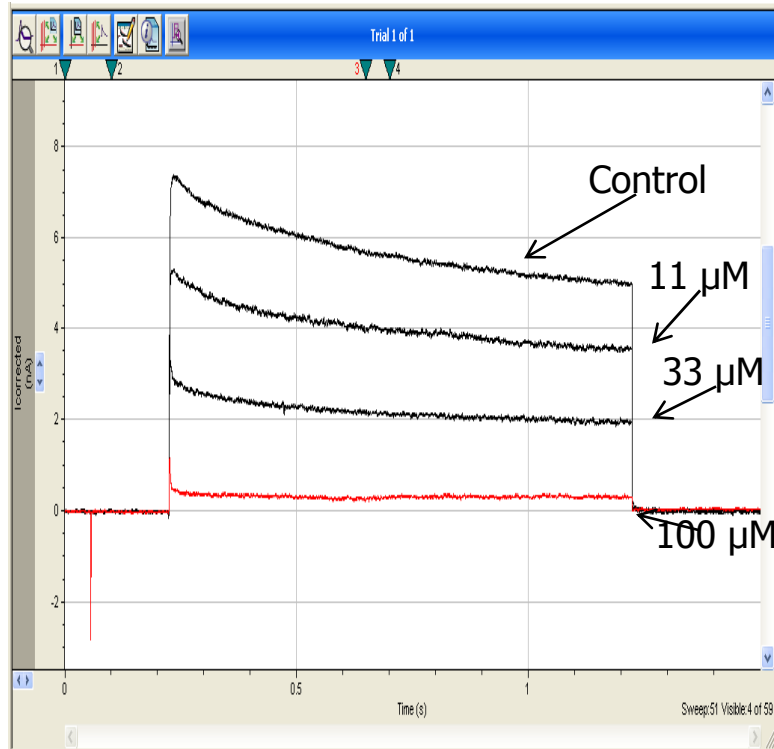


Compound	MaxCyte transfected cells	BioFocus stable cell line	Literature
	End Step IC ₅₀	End Step IC ₅₀	IC ₅₀
Capsaicin	12 μM	48 μM	23 μM
Nifedipine	10 μM	16 μM	27 μM
Bupivacaine	49 μM	66 μM	13 μM

PatchXpress[®] K_v1.5 Capsaicin Pharmacology

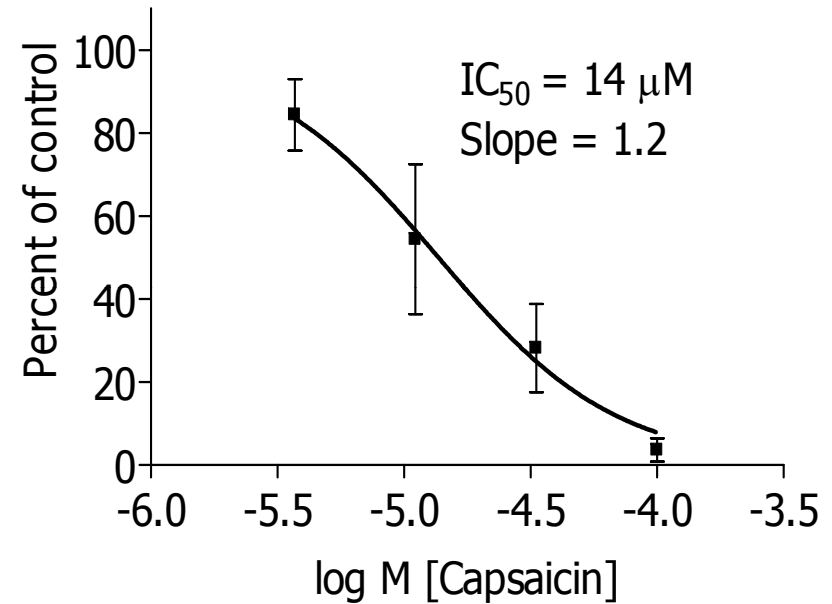


A.



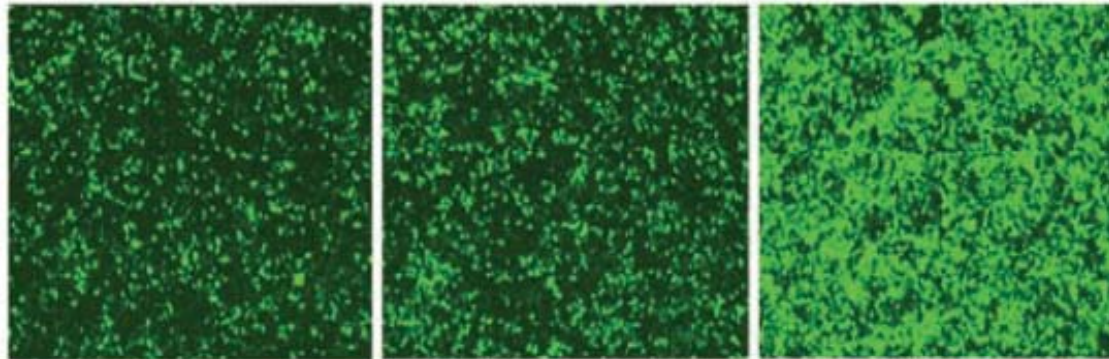
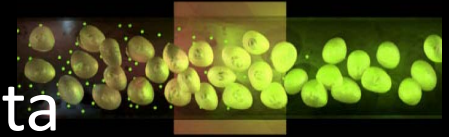
Data = mean \pm SD (n \geq 4 cells)

B.



- 2 SealChips
- 19 whole-cell recordings (possible 32)
- 17 (89%) recordings with current > 0.5nA
 - Comparable with single hole data
- Mean current amplitude 5.96 \pm 3.27nA

HEK Cells Transfected with $K_v1.3$ -GFP: GFP Expression and PatchXpress[®] Activity Data



100 µg/mL

200 µg/mL

300 µg/mL

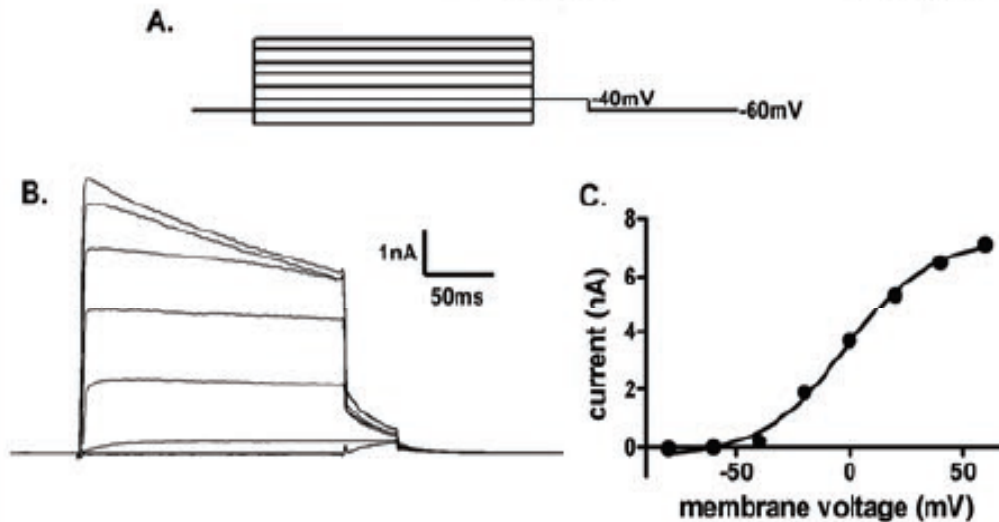


Fig 4. Recording of $K_v1.3$ currents in HEK-293 cells transfected with $K_v1.3$ -GFP plasmid DNA. HEK-293 cells were transfected with 300 µg/ml DNA and plated on 35mm culture dishes. Thirty-six to forty-eight hours post transfection, cells were trypsinized and resuspended immediately before they were assayed on the PatchXpress 7000A system. **A)** Voltage protocol; **B)** Sample $K_v1.3$ currents recorded from a transfected cell; **C)** Current-voltage relationship of $K_v1.3$ channel extracted from the same cell as in B; each data point represents the peak outward current elicited by its respective voltage step .

mTMEM16A Chloride Channel Activity in Transfected HEK Cells: PatchXpress® Data

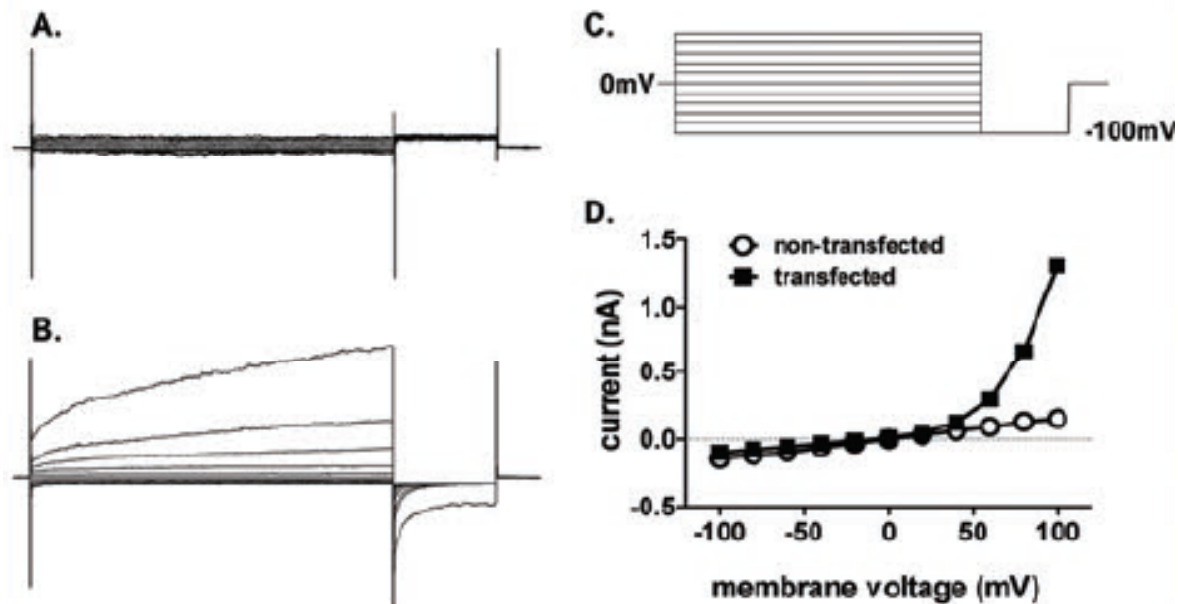
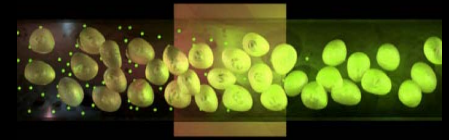
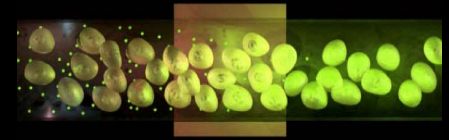


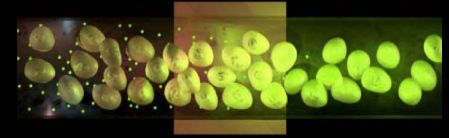
Fig 6. Recording of whole-cell currents in HEK-293 cells transfected with mTMEM16A plasmid DNA. HEK-293 cells were transfected with 300 $\mu\text{g}/\text{ml}$ DNA and plated on 35mm culture dishes. Thirty-six to forty-eight hours post transfection, cells were trypsinized and resuspended immediately before they were assayed on PatchXpress 7000A system. **A)** Sample recording from a HEK-293 parental cell showing only the leak currents; **B)** Sample recording of TMEM16A currents from a transfected cell; **C)** Voltage protocol ;**D)** Current-voltage relationships extracted from the same non-transfected parental cell as in A (open circles) , and transfected cell as in B (closed circles). Note significant outward rectification of current at depolarized potentials, a known characteristics of TMEM16A channel.

Summary



- Multiple plasmids can be co-transfected with the MaxCyte STX, enabling rapid screening of multi-subunit ion channels.
- STX transfected cells exhibit high functional expression on FLIPR^{TETRA}[®] and IonWorks[®] Quattro[™] in both single hole and population patch clamp modes .
- Expression levels and current readings of STX transfected cells are superior to those of lipid transfected cells.
- Cells can be transfected in bulk with ion channel plasmids using the MaxCyte STX and cryopreserved for future ion channel assays.
- STX transfected cells form giga-ohm seals, enabling rapid generation of pharmacology data with PatchXpress[®] and related screening platforms.

MaxCyte STX Advantages



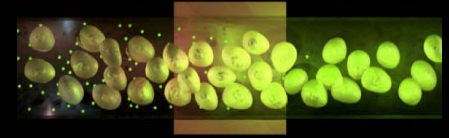
- Small to large volume scalability, ~10 billion cells in < 30 minutes
- Consistent and reproducible transfections
- High cell viability and transfection efficiency
- Transfection of multiple agents simultaneously
- Minimal off-target effects



- More relevant assays
- Faster experimental turnaround
- Greater productivity

Better Drug Candidates

Acknowledgements



The Ion Channel Company

- Yuri Kuryshev
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- Xin Jiang
- Jeff Netzeband
- David Yamane

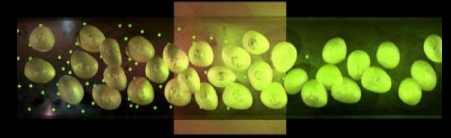


- Omar Aziz
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- Susan Gale
- Matt Gardener
- Tony Lawrence
- Scott Maidment
- Andrew Southan



- Cornell Allen
- Linhong Li
- Rama Shivakumar
- Angelia Viley





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