Development of Screening Technology Using Synthetic Lipid Vesicles & ICR

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Need for HTS

- **Ion Channels**
  - Important therapeutic targets
  - Biopesticide targets
- **Need for HTS Bio-assays**
  - Large chemical / biochemical libraries
    - Combinatorial chemistry
    - CAD modelling & designing
  - New ion channel targets
    - Genomics & proteomics
    - Disease models

Aurora Biomed's ICR Technology
An Overview

Aurora Biomed's ICR Series

- High throughput Analytical Instrument
  - ICR 8000

  - ICR 12000

Parameters	ICR 12000
Sensitivity	0.05mg/L (0.05ppm)
Sampling Volume	100 µL @ ~5% CV 50µL @ ≤10% CV
Throughput/ 8hr day	24,000 data points
Cost/well	≤ $0.04/well
Fuel	natural gas - compressed air
Add-on Options	included: plate stacker, bar code reader
Plate format	96/384-well Plates
Bench space required	120cm X 95cm

Surrogate Ions

- Potassium ion channels: Rb^+ for K^+
- Na^+,K^+ ATPase: Rb^+ for K^+
- Sodium ion channels: Li^+ for Na^+
- Acid sensing ion channels: Li^+ for Na^+
- Chloride ion channels:
  - Ag^+ titrated with Cl^- in the samples
  - Ag^+ precipitates as AgCl
  - Ag^+ remaining free is measured in the samples
- K^+,Cl^- co-transporter: Ag^+
- Calcium ion channels: Sr^{2+} or Ca^{2+}

Expression Systems

- Endogenously expression
- Stable or transient expression
  - Mammalian cell lines
    - HEK293 cells
    - Mouse L cells
    - CHO cells
  - Non-mammalian
    - *Xenopus* Oocytes
    - C. elegans
    - Yeast

Targets Screened Using ICR

Ion Channel	Associated Diseases
hERG	Long-QT syndrome, drug-induced arrhythmias
KCNA3 (Kv1.3)	Multiple sclerosis, obesity, diabetes
KCNQ2/3	Epilepsy
KCNA5 (Kv1.5)	Pulmonary hypertension
BK_{Ca}	Erectile dysfunction
SK_{Ca}	Incontinence
KCNA1 (Kv1.1)	Episodic ataxia
KCNA4 (Kv1.4)	Ventricular diseases

Targets Screened Using ICR (cont.)

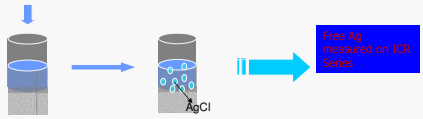
Ion Channel	Associated Diseases
Stretch-activated K^+ channels	Muscle damage
Na^+/K^+-ATPase	Congestive heart failure
K-Cl co-transporter	Sickle cell diseases
SCN5A ($Na_v1.5$)	Long-QT syndrome
SCN2A ($Na_v1.2$)	Multiple sclerosis, seizure disorders
CFTR	Cystic Fibrosis
Cl_{Ca}	Asthma

To date, these targets have been studied using ICR, and more are being developed and optimized

Chloride Determination

30ul Ag Solution (50 mg/L)

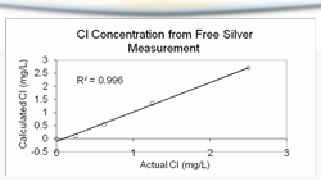
Precipitation of Cl- as AgCl



200ul Sample

Calculation of Sample: Cl=2.46-0.378Y+0.778/Y
where: Y=excess Ag concentration

Chloride Determination



200-µl Cl (ppm)	30-µl Ag (ppm)	Excess Ag (abs)	Excess Ag (Mean Abs)	Excess Ag (ppm)	calculated Cl (ppm)
0	0	0.038, -0.058, -0.051	-0.024	0	0
0	50	3.551, 3.026, 3.257	3.278	6.5	0
0.25	50	3.051, 3.051, 3.154	3.085	6.0	0.32
0.62	50	2.926, 2.593, 2.919	2.813	5.5	0.52
1.25	50	2.022, 2.036, 1.967	2.008	3.9	1.18
2.50	50	0.878, 0.908, 1.093	0.960	1.8	2.21

HTS and Nav1.7- Li Flux

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Cellular HTS Assays for Pharmacological Characterization of $Na_V1.7$ Modulators

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HTS and Nav1.7

- ICR12000 AAS-based Li-flux assay
- FLIPR membrane potential assay
- Fluorescense resonance energy transfer (FRET)-based membrane potential assay
- IonWorks EP

HTS and Nav1.7

TABLE 1. COMPARISON OF pIC_{50} OF KNOWN Na_V BLOCKERS IN THE MEMBRANE POTENTIAL ASSAY (FLIPR MEMBRANE POTENTIAL DYE [MPD] IN THE FLIPR AND FRET DYE PAIR IN THE HAMAMATSU ASSAY), LI-AAS INFLUX ASSAY, AND IONWORKS HT

	pIC_{50}			
	25 μM veratridine + 1 μg/ml SVqq			
	FLIPR (MPD blue dye)	*FRET (VSP dye pair)*	*AAS (Li influx)*	*IonWorks HT (current)*
Lidocaine	3.03	3.50	3.89	4.00
Bupivicaine	4.38	5.40	4.77	4.40
Quinidine	4.20	4.29	4.52	4.50
Flecainide	—	4.37	4.40	4.90
TTX	6.58	6.21	5.40	8.00
Flunarizine	5.58	5.35	5.59	6.70
Mexiletine	4.97	4.57	4.29	4.50
Phenytoin	5.03	4.81	4.19	4.50
Propafefenone	6.21	5.70	6.13	5.30
Tetracaine	6.00	5.76	5.26	6.55
CO-102862	5.70	5.06	5.21	>4.0

Compounds were tested in triplicate on two or three different days.

Li flux Assay Development

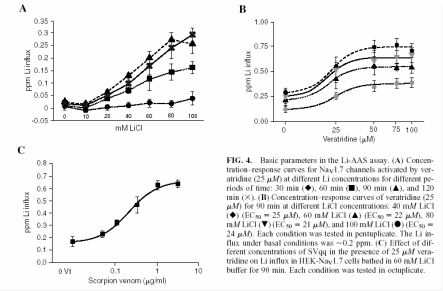

FIG. 4. Basic parameters in the Li-AAS assay. (**A**) Concentration–response curves for $Na_V1.7$ channels activated by veratridine (25 μM) at different Li concentrations for different periods of time: 30 min (◆), 60 min (■), 90 min (▲), and 120 min (×). (**B**) Concentration–response curves of veratridine (25 μM) for 90 min at different LiCl concentrations: 40 mM LiCl (◆) (EC_{50} = 25 μM), 60 mM LiCl (▲) (EC_{50} = 22 μM), 80 mM LiCl (▼) (EC_{50} = 21 μM), and 100 mM LiCl (●) (EC_{50} = 24 μM). Each condition was tested in pentaplicate. The Li influx under basal conditions was ~0.2 ppm. (**C**) Effect of different concentrations of SVqq in the presence of 25 μM veratridine on Li influx in HEK-$Na_V1.7$ cells bathed in 60 mM LiCl buffer for 90 min. Each condition was tested in octuplicate.

ICR12000 vs FLIPR

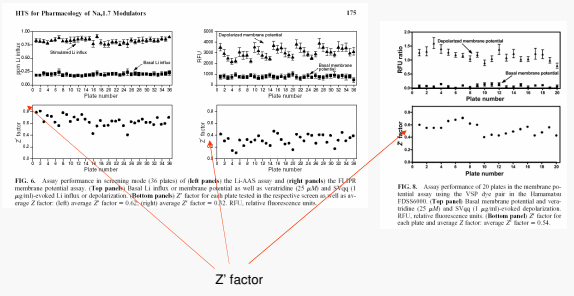

FIG. 6. Assay performance in screening mode (36 plates) of (**left panels**) the Li-AAS assay and (**right panels**) the FLIPR membrane potential assay. (**Top panels**) Basal Li influx or membrane potential as well as veratridine (25 μM) and SVqq (1 μg/ml)-evoked Li influx or depolarization. (**Bottom panels**) Z′ factor for each plate tested in the respective screen as well as average Z factor: (left) average Z′ factor = 0.62; (right) average Z′ factor = 0.52. RFU, relative fluorescence units.

FIG. 8. Assay performance of 20 plates in the membrane potential assay using the VSP dye pair in the Hamamatsu FDSS6000. (**Top panel**) Basal membrane potential and veratridine (25 μM) and SVqq (1 μg/ml)-evoked depolarization. RFU, relative fluorescence units. (**Bottom panel**) Z′ factor for each plate and average Z factor: average Z′ factor = 0.54.

Z' factor

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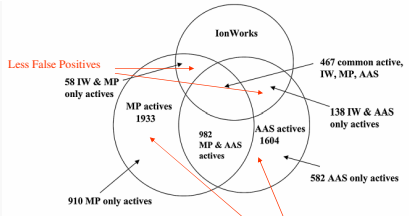

FIG. 7. Overlap of $Na_V1.7$ channel blockers in IonWorks HT (IW), Li-AAS (AAS), and membrane potential (MP) assays.

Correlation with IonWorks HT

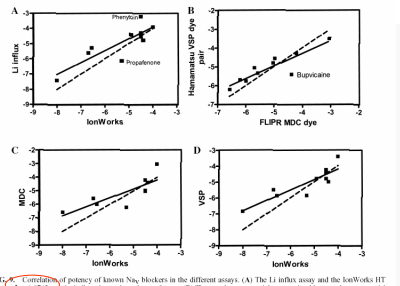

FIG. 5. Correlation of potency of known Na_V blockers in the different assays. (**A**) The Li influx assay and the IonWorks HT assay, r^2 = 0.8762, not including phenytoin and propafenone. (**B**) The membrane potential assay using blue membrane potential dye from Molecular Devices Corp. (MDC) in the FLIPR and the VSP dye pair in the FDSS6000, r^2 = 0.8037. (**C**) The IonWorks HT and membrane potential assay using blue membrane potential dye (MDC) in the FLIPR, r^2 = 0.6535. (**D**) The IonWorks HT and the membrane potential assay using the VSP dye pair in the FDSS6000, r^2 = 0.7695. The solid line is the line of correlation, and the dotted line is the line of correspondence.

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Cellular HTS Assays for Pharmacological Characterization of $Na_V1.7$ Modulators

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Abstract: Ion channels are challenging targets in the early phases of the drug discovery process, especially because of the lack of technologies available to screen large numbers of compounds in functionally relevant assays. The electrophysiological patch-clamp technique, which is the gold standard for studying ion channels, has low throughput and is not amenable to screening large numbers of compounds. However, for random high-throughput screening (HTS) of compounds against ion channel targets, a number of functional cellular assays have become available during the last few years. Here we use the sodium channel $Na_V1.7$ stably expressed in human embryonic kidney 293 cells and compare three HTS assays—a Li flux atomic absorption spectroscopy (AAS) assay, a fluorescent imaging plate reader (FLIPR™, Molecular Devices, Sunnyvale, CA) membrane potential assay, and a fluorescence resonance energy transfer (FRET)-based membrane potential assay—to an automated electrophysiological assay (the Ionworks™ HT [Molecular Devices] platform) and characterize 14 known Na_V inhibitors. Our results show that all three HTS assays are suitable for identification of $Na_V1.7$ inhibitors, but as an HTS assay the Li-AAS assay is more robust with higher Z′ values than the FLIPR and FRET-based membrane potential assays. Furthermore, there was a better correlation between the Ionworks assay and the Li-AAS assay regarding the potency of the Na_V inhibitors investigated. This paper describes the first comparison between all the HTS assays available today to study voltage-gated Na_Vs, and the results suggest that the Li-AAS assay is more suited as a first HTS assay when starting an Na_V drug discovery campaign.

New Applications

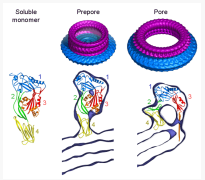
Objectives

- To study pharmacology of ion channel targets in isolation from
  - Endogenous ion channels
  - Backgrounds
- Homologous or heterologous expression systems that do not provide adequate signal for ICR
- The ions that cannot be analyzed by AAS based ICR
  - H^+ channels influenza virus
- To study pore forming ion channels

Pore Forming Proteins

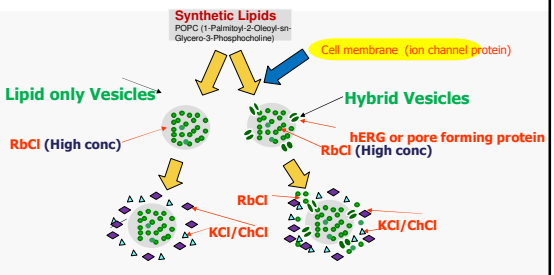
- Pore forming proteins
  - Examples

Viruses	Sendai, Newcastle Disease, Influenza
Bacterial toxins	S. aureus α- and δ-toxin, Streptolysin O, C. perfringens θ-toxin, S. pneumoniae pneumolysin, E. coli haemolysin, *A. hydrophobia* haemolysin, C. lactens cytolysin, B. thuringiensis δ-endotoxin
Animal toxins	Melittin (honey bee), Cytolysin (sea anemone), Latrotoxin (spider venom)
Immune proteins	Activated complement, Cytolysin (perforin)
Synthetic compounds	Polycations, Triton X-100



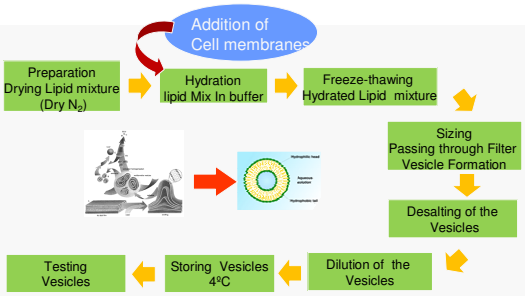
Concept of using Synthetic Lipid Vesicles

Lipid Vesicle Assay- Principle



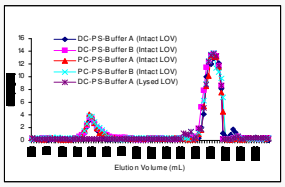
Preparation & Characterization of Lipid Only Vesicles

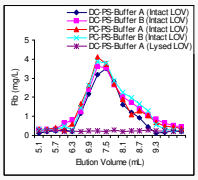
Preparation of Vesicles



Desalting LOV- Sepharose Column

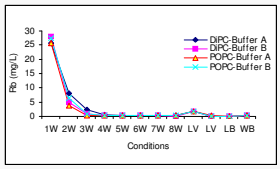
- LOV (0.8 μm, 10mg/mL lipid)
- LOV (100uL) using Sepharose-4B column:
  - Rb^+ Profile
  - The LOV elution peak
  - Absence of LOV from lysed LOV sample
- Indicates entrapment of Rb in the vesicles

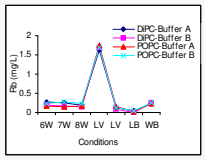




Desalting LOV- Spin Microplates

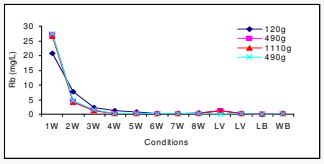
- LOV (0.8 μm, 10mg/mL lipid)
- Bio-Inert 96-well Spin Microplates:
  - Rb^+ profile
    - Intact LOV vs lysed vesicles
  - Intra-LOV Rb^+ content
    - In different buffers
    - Contain almost the same amount of Rb^+

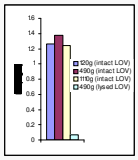




Desalting LOV- Spin Microplates

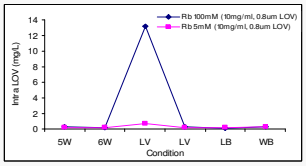
- Optimization-centrifugation
- Rb^+ profile the steps
- Intra-LOV content of Rb^+
  - Showing RCF of 490g adequate





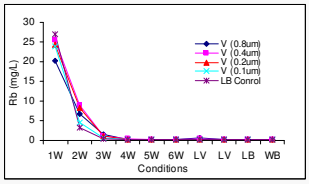
Optimization of Rb^+ in LOV

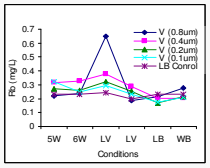
- Optimization of Rb^+ for LOV preparation
  - 5 mM
  - 100 mM



Optimization- LOV size

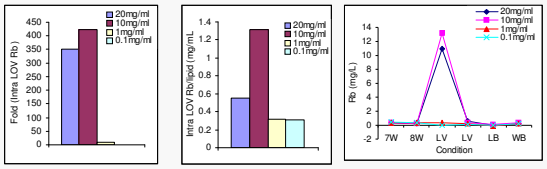
- Rb^+ profile
  - Different sized LOV
- Rb^+ content peak
  - Different size LOV in comparison
  - LB (lysis buffer)





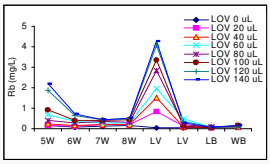
Optimization – Intra Rb+ Content

- Intra-LOV Rb+ content
- Intra-LOV Rb+/lipid (mg/mL)
  - Fold intra-LOV Rb+ content



Optimization – Volume of LOV

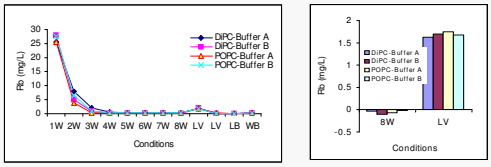
- Optimization - liquid volume of LOV for use
  - 0, 20, 40, 60, 80, 100, 120, 140 uL
  - Adequate 40uL



Leakage Test of LOV

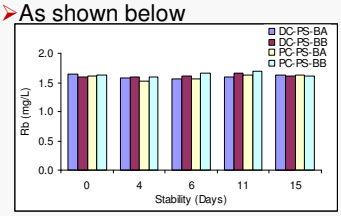
Leakage of LOV- Lipid compositions

- Leakage profile LOV
  - LOV-different lipid compositions
  - Rb+ profile of the desalting
  - Leakage of LOV



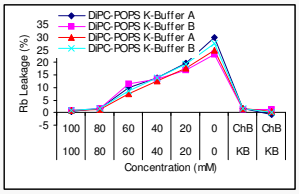
Leakage & Shelf Life of LOV

- Leakage & stability of LOV (0.8 µm, 10mg/mL)
  - As shown below



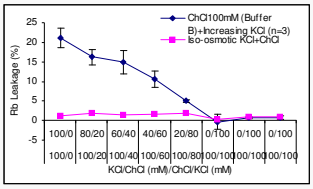
Rb+ Leakage –Different Conditions

- Rb+ leakage during
  - Hypo-, hyper or iso-osmotics buffers
    - Decreasing concentration of KCl
      - In K+-Buffer A/B or
      - Decreasing concentration of choline in choline Buffer A/B



Rb^+ Leakage –Different Conditions

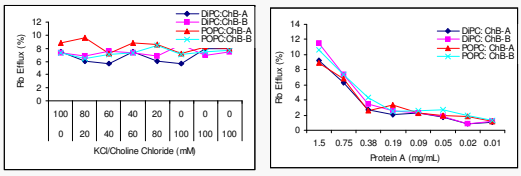
- Rb^+ leakage during
  - Comparing
    - controlled trans-membrane potential
    - Osmolarity



LOV & Pore Forming Protein Interaction

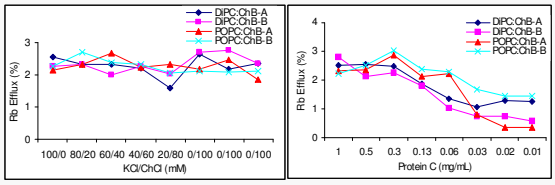
Rb^+ Leakage- Protein A Interaction

- Rb^+ leakage from interacting protein A
  - 1.5 mg/mL of protein in the iso-osmotic KCl+ChCl buffer A/B.
  - Various concentrations of protein 1 in presence of choline buffer A/B.



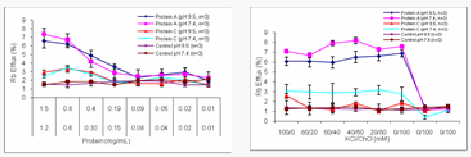
Rb^+ Leakage- Protein C Interaction

- Rb^+ leakage from LOV-protein C interaction
  - Protein(1mg/mL)
    - In iso-osmotic KCl + ChCl buffer A/B
- Varying concentrations of protein
  - In presence of Choline Buffer A/B



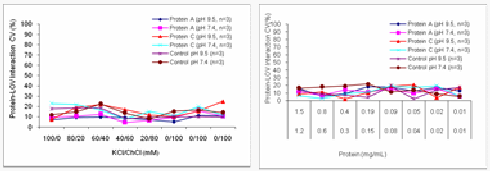
Rb^+ Leakage (n=3)

- Rb^+ leakage among the replicates (n=3)
- Protein A (1.5 mg/mL) or C (1.2 mg/mL)
  - In presence of varying LOV potential (iso-osmotic KCl+ChCl buffer A/B)
- Varying concentrations of proteins A or C in presence of Choline Buffer A/B.



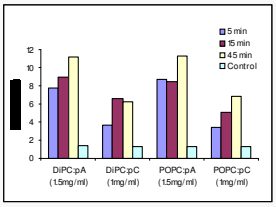
CV (n=3) LOV- Protein Interactions

- CV (%) among the replicates (n=3)
- LOV to protein A & C
  - Varying LOV potential (iso-osmotic KCl+ChCl buffer A/B)
- LOV to various concentrations of the proteins A & C
  - In presence of Choline Buffer A/B



Optimization- Period of Efflux

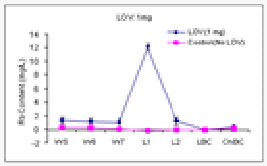
- Optimization of time of efflux
  - Incubation with protein A or C for long duration
    - No significantly influence on Rb^+ leakage from LOV



Hybrid Vesicles

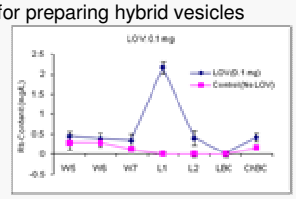
Optimized Assay- Hybrid Vesicles

- LOV (1 mg/mL, 0.8um, Intra LOV RbCl 300 mM, Ph7.5)
- Wash Buffer: Isotnic solution
- Activation Buffer: ChCl 300 mM, Ph7.5
- Activation (n=8)
  - Intense signal for ICR
  - Insignificant leakage of RB
- Better use 0.1 mg lipid rather than 1 mg lipid for preparing hybrid



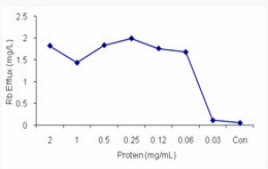
Optimized Assay- Hybrid Vesicles

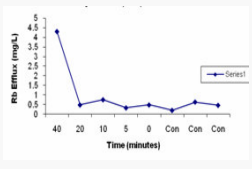
- LOV (0.1 mg/mL, 0.8um, Intra LOV RbCl 300 mM)
- Wash Buffer: Isotonic (Ph7.5)
- Activation (n=8)
  - Significant amount of signal expected
    - Insignificant leakage of RB (W7)
    - Lysis: Showing about hundred % release of Rb^+
    - Insignificant leakage of Rb^+
- Can be used for preparing hybrid vesicles



Optimized Assay- Hybrid Vesicles

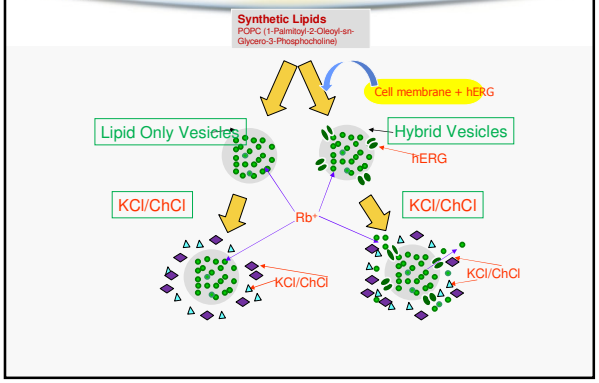
- HV (0.1 mg/mL, Ratio of Lipid: Biological target membrane =1:1, 0.8um)
- HV used in assay: 40 uL of 10X diluted from original preparation
- Intra HV Buffer: RbCl 300 mM (Ph7.5)
- Wash Buffer isotonic
- Activation Buffer
  - ChCl 300 mM + Protein C
  - Period of activation optimized
  - Adequate signal for ICR
  - Insignificant leakage of RB in the control (con)



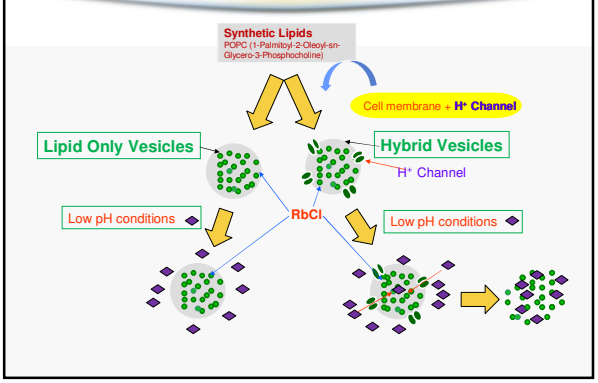


Applications

Potential Applications



Potential Applications



CONCLUSION

- ICR technology is applicable
  - Diverse channels
    - K^+, Na^+, Ca^{2+}, Cl^-
    - Pore forming proteins
- Continue to develop new applications for ICR
- Automation of the assays