Generation of *Drosophila* cell lines expressing mammalian Orai3 and STIM1 for drug discovery

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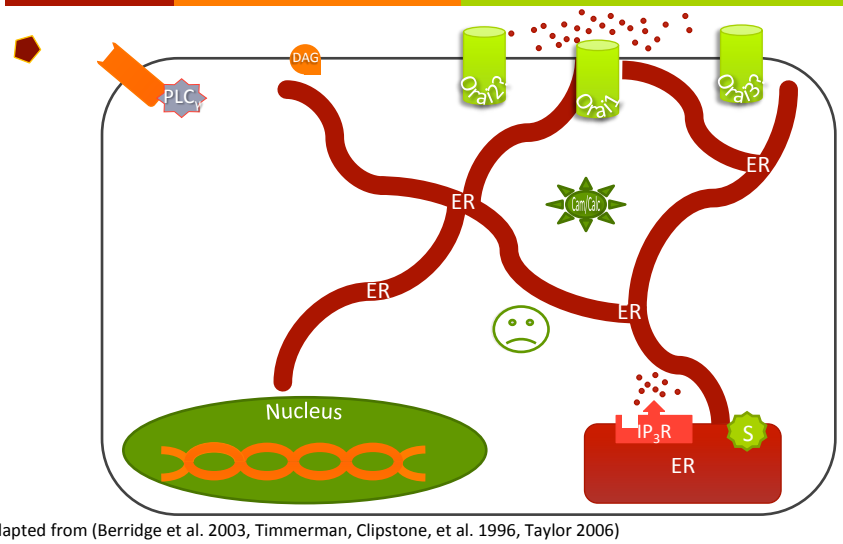
In the beginning, there was Ca^{2+}

- Ca^{2+} is a critical cellular **signaling molecule** (Berridge et al. 2000, Berridge et al. 2003).
- Maintaining intracellular Ca^{2+} **homeostasis** is critical for continued survival.
  - SCID is an example of what happens when Ca^{2+} homeostasis is upset. (Feske et al. 2006)
- One method cells use to control cellular free Ca^{2+} is **store-operated calcium entry** (SOCE).

Background: SOCE

- In **response to depleted endoplasmic reticulum (ER) Ca^{2+} stores**, extracellular Ca^{2+} is brought into the cell. (Vig et al. 2006, Smyth et al. 2010)
- **Orai1** is the **Ca^{2+} channel** responsible for SOCE. (Vig et al. 2006, Smyth et al. 2010)
- **Stim1** is the **Ca^{2+} sensor**. (Roos et al. 2005, Zhang, Kozak et al. 2008)
- Orai3 has sequence homology (49.0%) with Orai1, but its function in SOCE is **not clearly defined**. (Roos et al. 2005, Vig et al. 2006, Smyth et al. 2010)

Background: SOCE Mechanism



Adapted from (Berridge et al. 2003, Timmerman, Clipstone, et al. 1996, Taylor 2006)

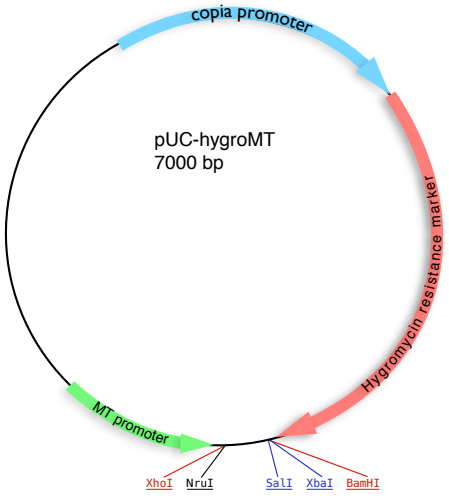
Background: Major Players

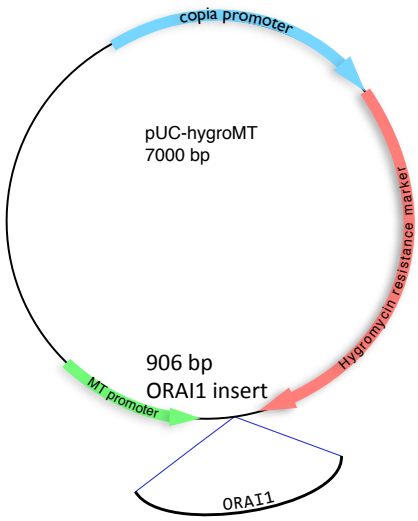
- **2-APB** – inhibits Orai1 at high concentrations and activates Orai1 at low concentrations
  (Prakriya & Lewis 2001, Goto et al. 2010)
- **Orai1** – ion channel component of SOCE
  (Berridge et al. 2000, Berridge et al. 2003)
- **Orai3** – Ca^{2+} channel related to Orai1, which is activated by 2-APB (Zhang, Kozak et al. 2008, Lis et al. 2007)

Specific Aims

- Create constructs which **express Orai3** and **Stim1**
- Prove that we can **express heterologous genes** in **S2 cells** (Schneider 1972, Johansen et al. 1989, Schetz et al. 2004)
- Perform experiments to **assess the effect** of 2-APB on heterologously expressed Orai3 **ion channels**

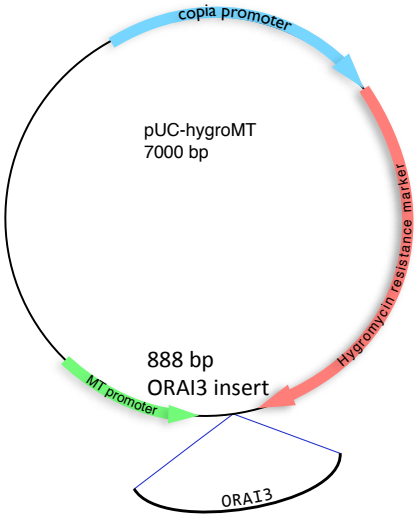
Methods: puc-HygroMT vector map

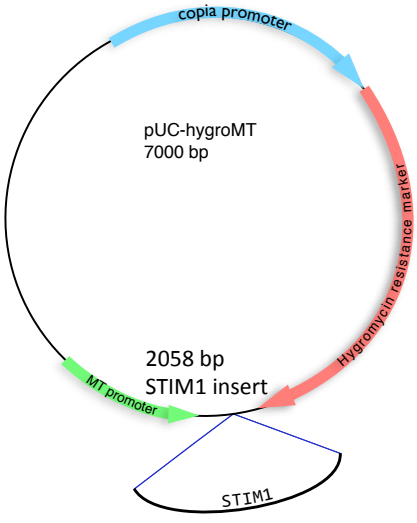




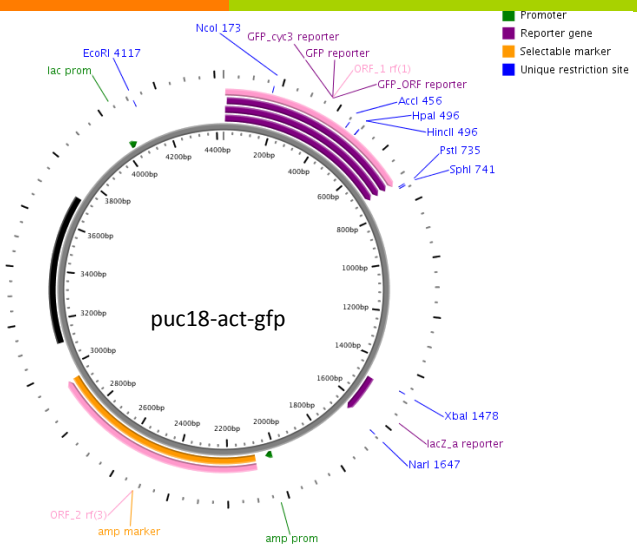
(Johansen et al., Schetz et al.)

Methods: Vector constructs

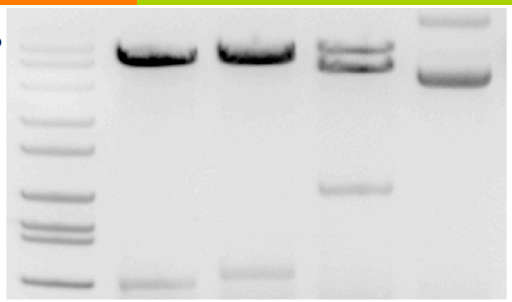




Methods: GFP Vector

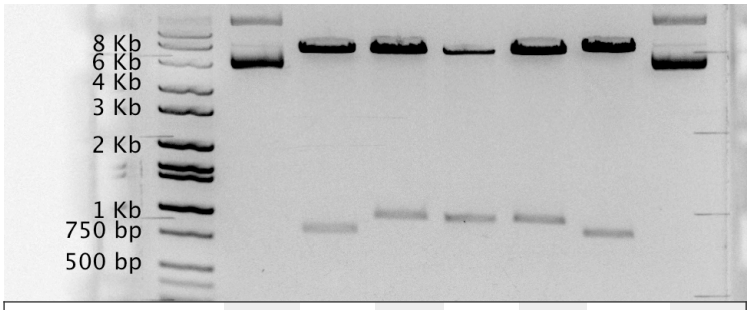


Results: Restriction digest of pucHygMT-Stim1



pucHygMT-Stim1		+	+	+	+
SalI + XbaI		–	–	+	–
BamHI + StuI		–	+	–	–
StuI + XhoI		+	–	–	–

Results: Restriction digest of pucHygMT-ORAI3 and pucHygMT-ORAI1

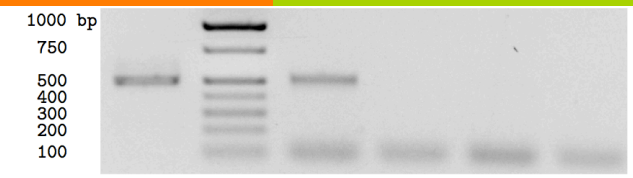


ORAI3				+++			
pucHygMT				+++			
pucHygMT-ORAI1	+++	+++	+++				
pucHygMT-ORAI3					+++	+++	+++
StuI + XbaI - ~900 bp			+++	+++			
ApaI + XbaI - ~780 bp		+++					
KpnI + XhoI - ~780 bp						+++	
StuI + BamHI - ~890 bp					+++		

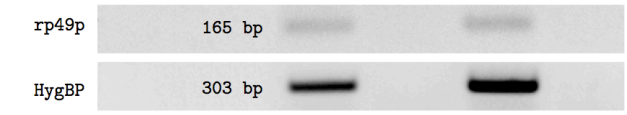
Results: RT-PCR of Orai3 after $CuSO_4$ induction

Orai3 RT-PCR band expected at 503 bp.
No band seen in no-RT lanes.

Faint bands visible around 100 bp, likely primers and/or incomplete PCR products



30 cycles



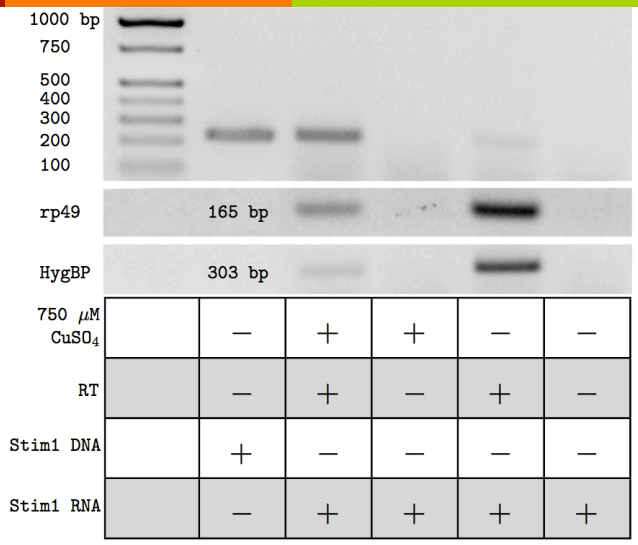
750 μM $CuSO_4$	−		+	+	−	−
RT	−		+	−	+	−
Orai3 DNA	+		−	−	−	−
Orai3 RNA	−		+	+	+	+

Results: RT-PCR of Stim1 after $CuSO_4$ induction

Stim1 RT-PCR band expected at 228 bp.
No band seen in no-RT lanes.

More DNA in Stim1 HygBP and rp49 PCRS from cells that were not induced.

Likely from errors in nanodrop when estimating concentration.

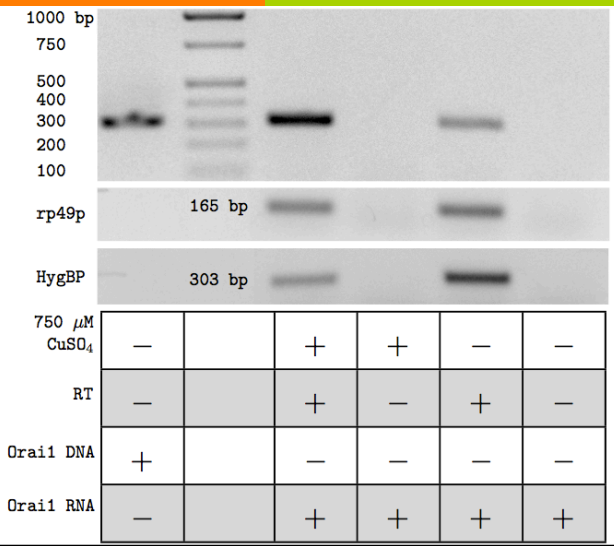


Results: RT-PCR of Orai1 after $CuSO_4$ induction

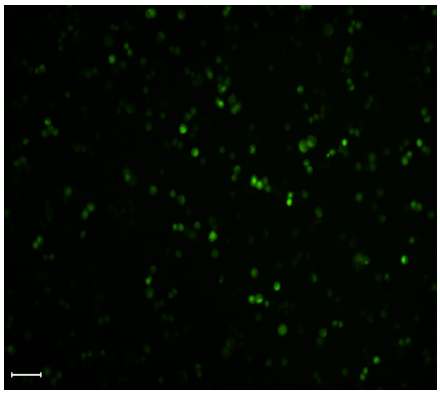
Orai1 RT-PCR band expected at 311 bp.
No band seen in no-RT lanes.

More DNA in Orai1 HygBP PCRs from cells that were not induced.

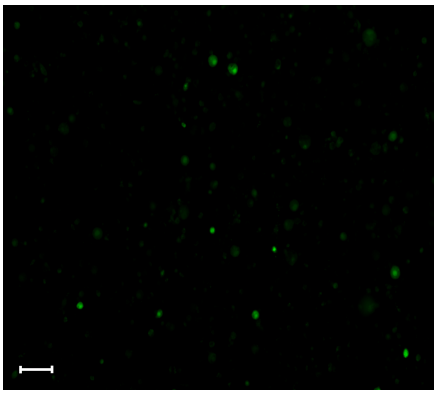
Likely from errors pipetting.



Results: GFP Transfected S2 cells



Transfected S2 cells – 20X mag:
puc18-act-gfp – 2 µg
Bar: 50 µm



Transfected S2 cells – 20X mag:
pucHygMT-STIM1 – 2 µg
puc18-act-gfp – .25 µg

Summary #1

- Created constructs and verified identity
- Induced expression of heterologous Orai1, Orai3, and Stim1 genes in S2 cells using $CuSO_4$
- Expressed heterologous GFP in S2 cells
- Next – **Assess effect of orai3 ion channel expression in S2 cells**

Methods: Chemical reagents

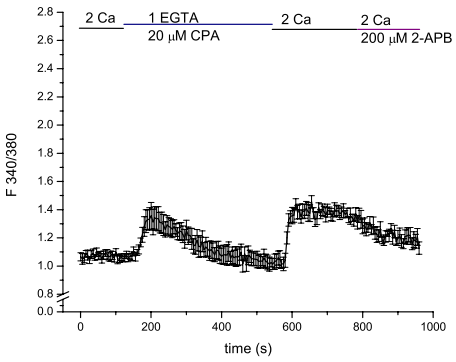
- **Fura-2** – fluorescent Ca^{2+} binding dye which allows monitoring of intracellular Ca^{2+} (Cordova et al. 2003, Lambert 2006)
- **Fura-2 AM** – membrane permeant version of Fura-2 (Cordova et al. 2003 , Lambert 2006)
- **Probenecid** – anion transport inhibitor which prevents cells from clearing Fura2 once inside cell. (Di Virgilio et al. 1990, Cordova et al. 2003)
- **Cyclopiazonic Acid** – inhibitor of SERCA pump. Result is leak of Ca^{2+} from the Endoplasmic Reticulum (ER) into the cytoplasm. (Putney 2006)

Methods: Measuring SOCE in S2s

- Incubate S2 cells with an S2 Ringer solution (McGuigan et al. 1991)
  - **2 Ca**: 2 mM $CaCl_2$, 5 mM KCl, 150 mM NaCl, 4 mM $MgCl_2$, 10 mM Dextrose, 10 mM HEPES, pH 7.2
  - **1 EGTA**: 1 mM EGTA, 5 mM KCl, 150 mM NaCl, 6 mM $MgCl_2$, 10 mM Dextrose, 10 mM HEPES, pH 7.2
- Dye loading solution:
  - **2 Ca** + 0.02% Pluronic F-127 + 2.5 mM Probenecid + 4 µM Fura2-AM

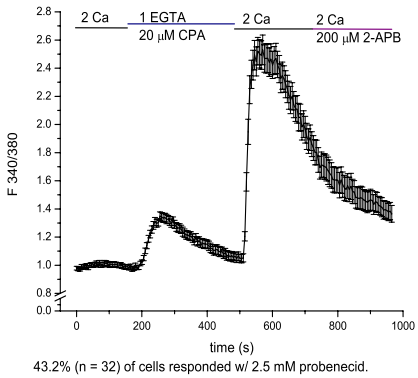
Results: Ca^{2+} measurements improved by addition of Probenecid

Effective measurement of SOCE is reduced in absence of Probenecid



Only 10.5% (n = 6) of cells responded without probenecid.

Measurement of SOCE is improved by addition of 2.5 mM Probenecid

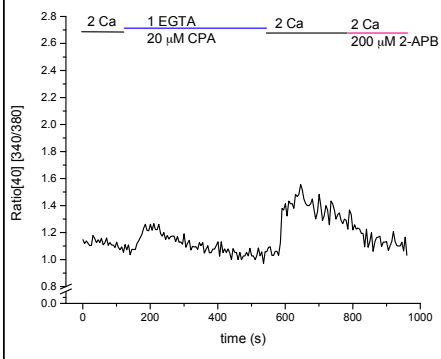


43.2% (n = 32) of cells responded w/ 2.5 mM probenecid.

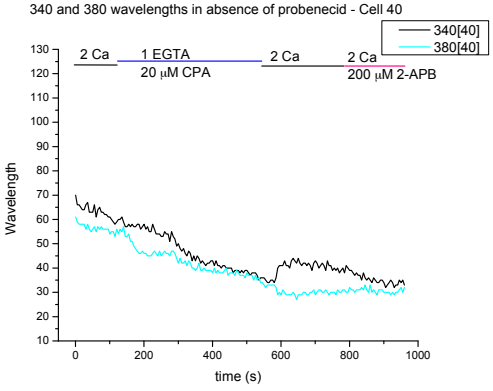
- Measuring the cytoplasmic Ca^{2+} of a population of S2 cells is improved by the addition of probenecid

Results: Sample trace of single cell in absence of Probenecid

Effective measurement of SOCE is reduced in absence of Probenecid

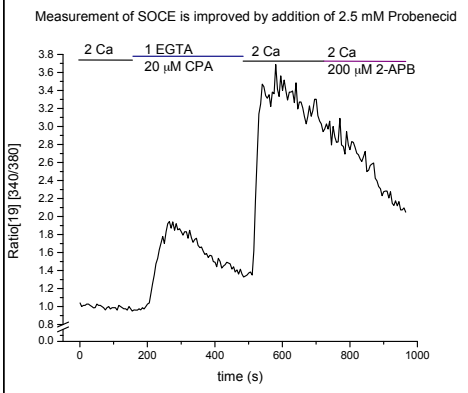


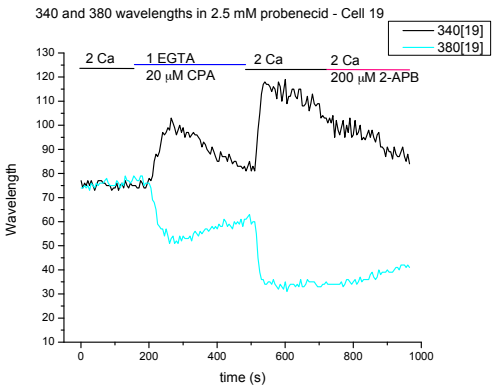
340 and 380 wavelengths in absence of probenecid - Cell 40



- In the absence of probenecid, we observe the gradual decrease in 340 nm and 380 nm values due to leakage of Fura2 dye from the cell.

Results: Sample trace of single cell when loading with 2.5 mM Probenecid



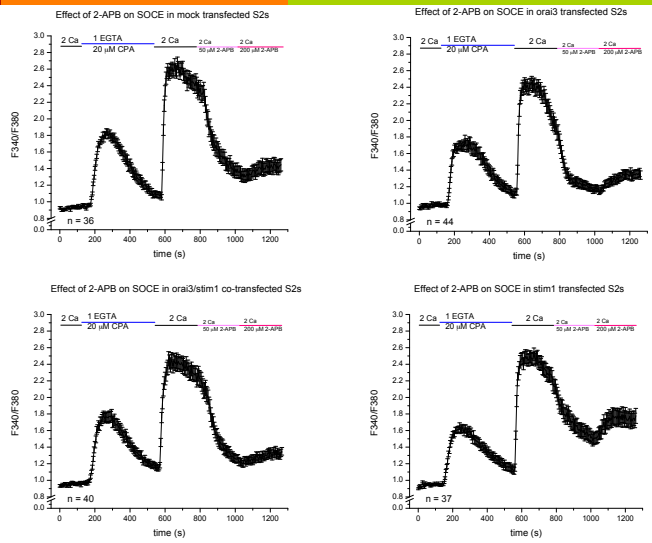


- With probenecid, we observe more robust 340 nm and 380 nm values, which do not suffer from issues with Fura2 dye leakage.
- Also we are better able to observe the increase in 340 and corresponding decrease in 380 wavelengths

Results: Data analysis

- Cells selected for analysis had to meet the following criteria:
  - 340 nm and 380 nm wavelength value remained above 40 during the initial 2 Ca perfusion.
  - CPA induced Ca^{2+} transient was visible.

Results: Variable Ca^{2+} responses with 2-APB in transfected S2 cells



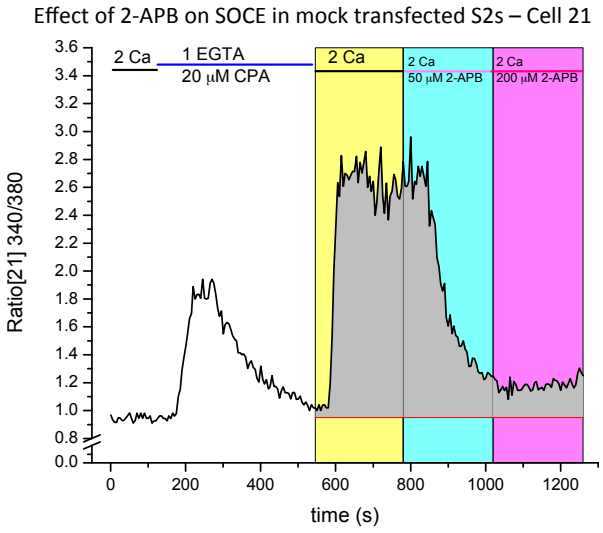
Results: Statistical Analysis

- Our null hypothesis (H_0) states that all the means are the same.
- If the p-value is smaller than our chosen significance level of 0.05, we conclude that the H_0 is not true.
  - Using 0.05 significance level means that we will have no more than a 5% chance of rejecting a true H_0
- In rejecting H_0, we conclude that at least one of the means is significantly different from the others.
- A subsequent post-hoc test will be used to identify where significant differences lie.

Results: Statistical Analysis

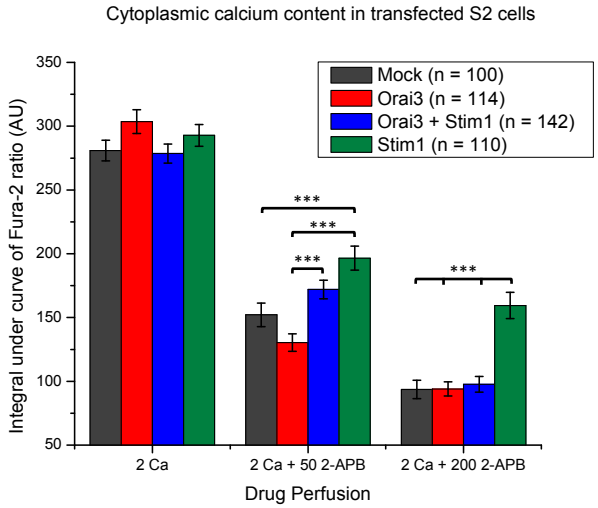
- Area under the curve (AUC) analysis was performed on the portion of the traces which followed CPA depletion of the ER.
  - **2 Ca**, **2 Ca + 50 µM 2-APB** and **2 Ca + 200 µM 2-APB** sections underwent AUC analysis, for 4 minutes each.
- A one-way ANOVA was performed on the AUC data to determine if there were significant differences between the perfusion group listed above.
- If significant differences were found, a Tukey's range test was performed on the group, to see which differences were significant between transfected groups:
  - i.e, mock, orai3, orai3 & stim1, and stim1

Results: Sample trace showing selections for area under the curve analysis



Results: Area under the curve analysis of cytoplasmic Ca^{2+} content

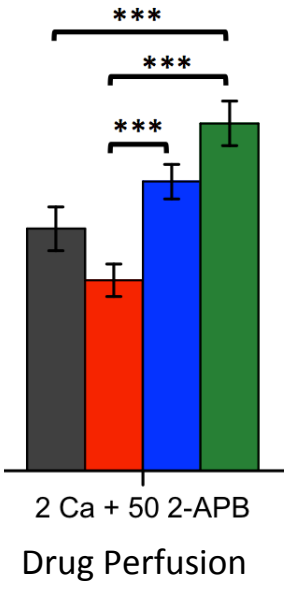
- One-way ANOVA shows no significant difference between the transfections, when only perfused with **2 Ca**
  - p = 0.1202
- There are significant differences between the transfected cells when perfused with **2 Ca + 50 µM 2-APB**, and also with **2 Ca + 200 µM 2-APB**



Results: Statistical analysis on 2 Ca + 50 µM 2-APB perfusion

Comparisons significant at the 0.05 level are indicated by ***.				
2 Ca + 50 µM 2-APB Comparison Groups	Difference Between Means	Simultaneous 95% Confidence Limits		
orai3+stim1 - orai3	41.59	13.22	69.97	***
stim1 - mock	44.46	13.29	75.63	***
stim1 - orai3	66.21	36.05	96.36	***

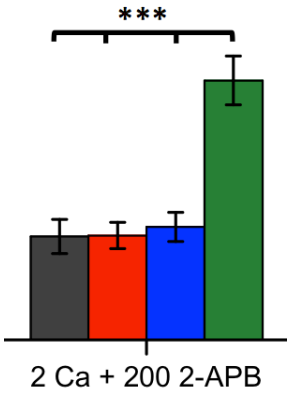
- One-way ANOVA, indicated a significant difference among the **2 Ca + 50 µM 2-APB** group.
  - p < 0.0001
- Subsequent Tukey's range test analysis, (above) indicated significant differences between:
  - Orai3 + Stim1 and Orai3 only transfection
  - Stim1 and mock transfections
  - Stim1 only and Orai3 only transfections



Results: Statistical analysis on 2 Ca + 200 µM 2-APB perfusion

Comparisons significant at the 0.05 level are indicated by ***.				
2 Ca + 200 µM 2-APB Comparison Groups	Difference Between Means	Simultaneous 95% Confidence Limits		
stim1 - orai3+stim1	61.793	35.560	88.026	***
stim1 - orai3	65.456	37.853	93.060	***
stim1 - mock	65.895	37.358	94.432	***

- One-way ANOVA, indicated a significant difference among the **2 Ca + 200 µM 2-APB** group
  - $p < 0.0001$
- Subsequent Tukey's range test analysis, (above) indicated significant differences between:
  - Stim1 only and Orai3 + Stim1 transfections
  - Stim1 and Orai3 only transfections
  - Stim1 and mock transfections



Summary #2

- Probenecid is necessary for effective recording of intracellular Ca^{2+} in S2 cells.
- S2 cells transfected with mammalian **Stim1** or **Orai3 & Stim1** show a significant increase in cytoplasmic Ca^{2+} content compared to **Orai3 only** transfected S2s, when treated with 50 µM 2-APB.
- S2 cells transfected with mammalian **Stim1** show a significant increase in cytoplasmic Ca^{2+} content compared to **mock** transfected S2s, when treated with 50 µM 2-APB.
- This suggests that **mammalian Stim1 is responsible** for the cytoplasmic Ca^{2+} increases observed.
- The fact that no significant difference was found when perfused with 2 Ca only, suggests that store depletion alone can not activate **Orai3 & Stim1** in S2s.

Summary #2 cont'd

- S2 cells transfected with mammalian **Stim1** show significantly higher cytoplasmic Ca^{2+} compared to **mock**, **Orai3** or **Orai3 & Stim1** transfected S2s, when treated with 200 µM 2-APB.
- The data suggest that **mammalian Stim1** takes part in processes which *slow the Ca^{2+} channel inactivation*. This results in a significant increase of intracellular calcium, when compared to the mock and other transfections.
- As is, our S2 expression system was unsuccessful in identifying an Orai3 specific response to 2-APB.

Future Work

- Use RNA interference to knock down native *dStim* and *dOrai* expression
  - This will allow us to look at the effect which mammalian Orai3 and Stim1 have, without the background of the native channels, as they may be contributing to effects observed.
- Generate stable cell lines to facilitate RNAi knockdown experiments

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