Fighting Alzheimer's: Fluorescent Indicators & Cell Perforation

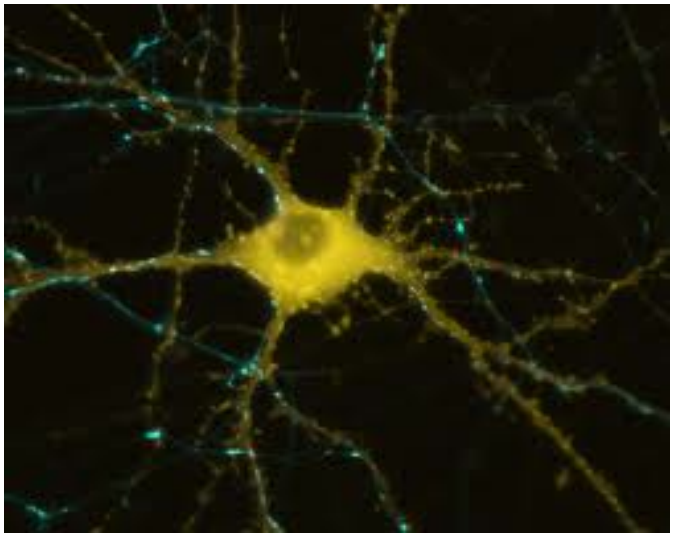
Taylor Allen
Gafni/Steel Lab
June – Aug 2011

Alzheimer's Disease (AD)

- Form of Dementia



- Problems with memory, behavior, thinking
- Most common (50%-80%)
- 6th leading cause of death
- Debilitating… eventually impairs motor functions
- De-conditioning… death

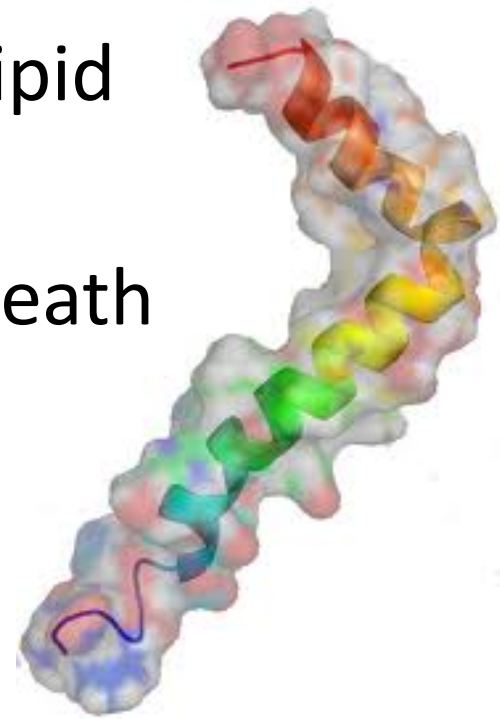


Alzheimer's Association: alz.org

Beta-Amyloid Background

- Small hydrophobic peptide
- Interacts w/ hydrophobic tails of lipid membranes
- Has been linked to neuronal cell death

Asp - Ala - Glu - Phe - Arg - His - Asp - Ser - Gly - Tyr - Glu - Val - His - His - Gln - Lys - Leu - Val - Phe - Phe - Ala - Glu - Asp - Val - Gly - Ser - Asn - Lys - Gly - Ala - Ile - Ile - Gly - Leu - Met - Val - Gly - Gly - Val - Val - Ile - Ala



Shaked, G.M, Kummer, M.P. (2007) NIH(Public access)

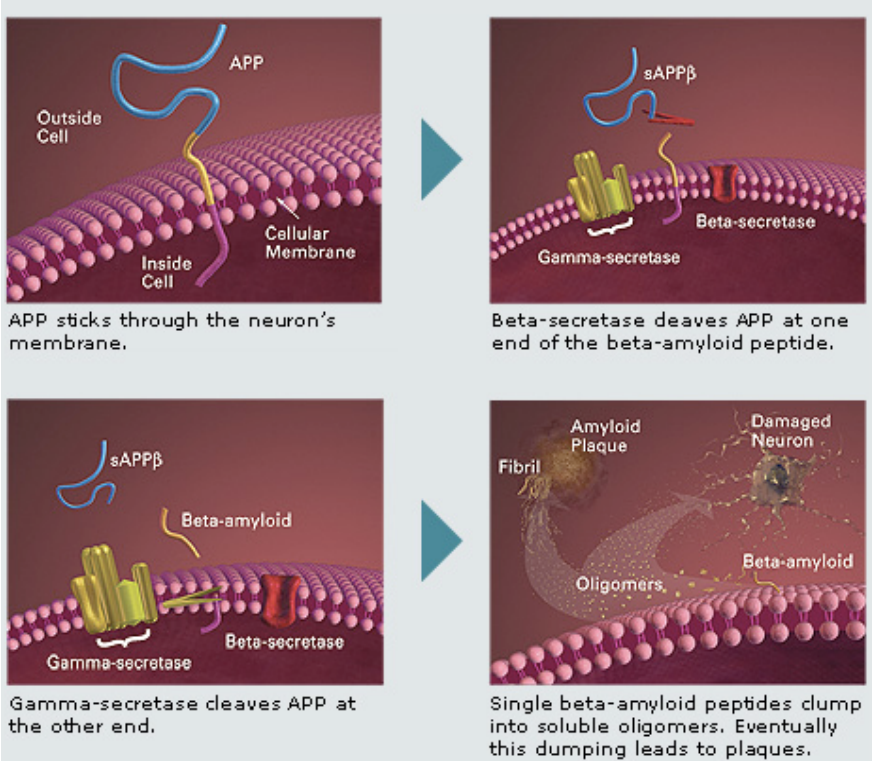
Background Aß

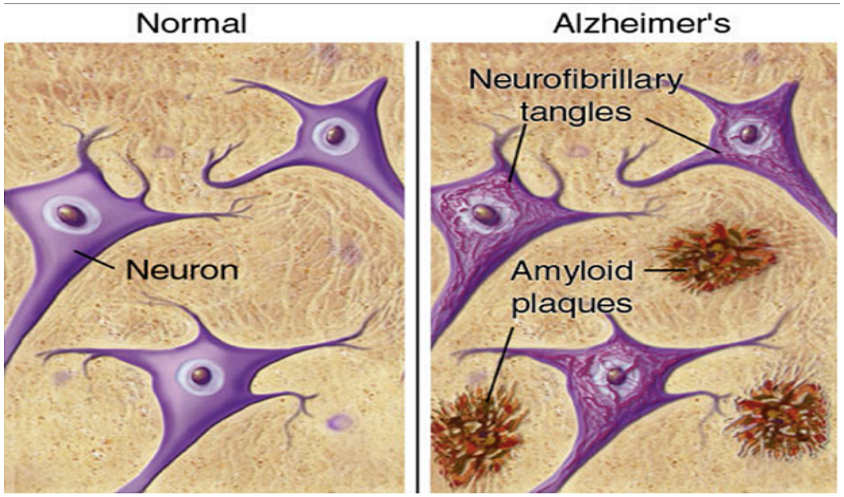
- Amyloid precursor protein (APP)
- Cleaved by secretase
- Releases Aß into space

  - Oligomerization & aggregation

  - Cell perforation, Ion homeostasis disruption, cell death

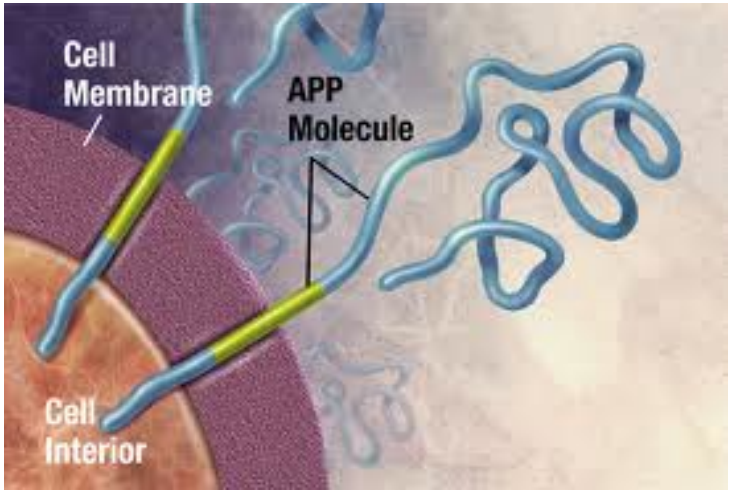
  - Fibril formation & plaques

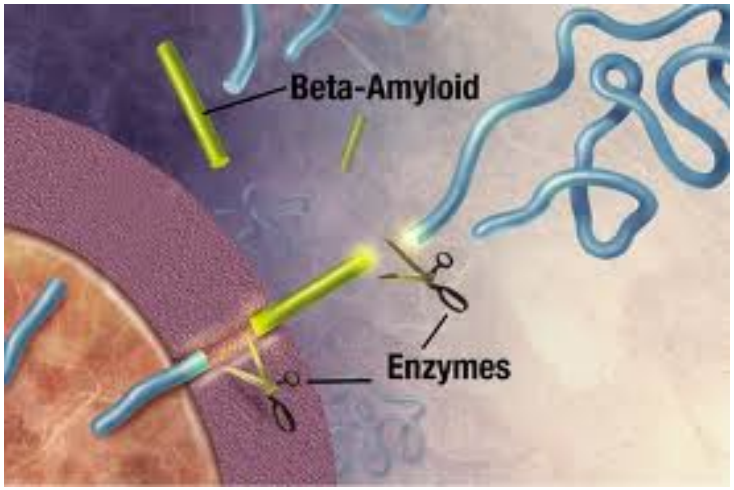




Shaked, G.M, Kummer, M.P. (2007) NIH(Public access)

Background Aß



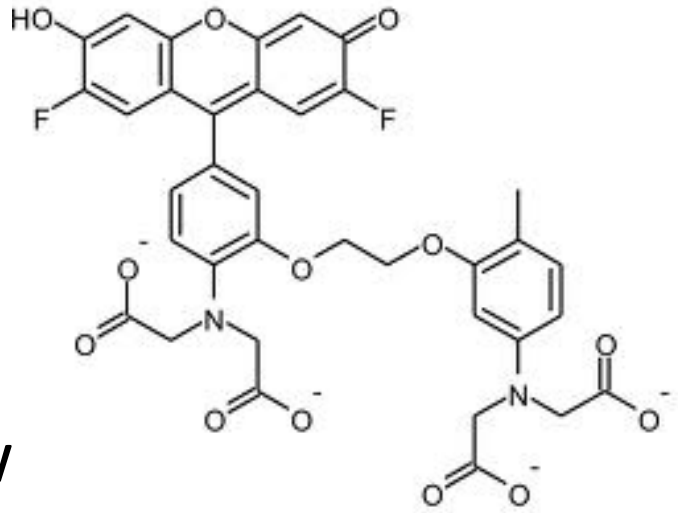


- Types associated w/ AD
  - 40 length peptide
  - 42 length peptide
- Aß40
  - Most common length peptide
- Aß42
  - Less common
  - More toxic (faster aggregation)

Shaked, G.M, Kummer, M.P. (2007) NIH(Public access)

Background… Calcium Indicator

- Aß perforations disrupt ion homeostasis (Ca^{2+})
- Fluo calcium indicators allow for imaging and characterizing of Ca^{2+} signaling and leakage
- Loading: Uncharged acetoxymethyl (AM) esters allow dye to permeate, which are cleaved by esterases to load the cell
- Objective: Induce fluorescence and Ca^{2+} signaling, characterize signaling & perforation



Molecular ProbesTM: Product Information, Fluo Calcium Indicators

Protocol

- Aliquots:

  1) 50mL HBSS (washing)

  2) 10mL HBSS (Fluo4)

→ Water bath at 37°C for 15 min

- Bring up Fluo4 (3µM), Aß42 (1µM), Ionomycin (60µM – 1:10), Buffer (500µL)
- 1) Wash cells with ~ 2mL of HBSS

  2)Incubate with ~ 2mL of Fluo4 for 15 min

  3) Wash twice more with ~ 2mL of HBSS

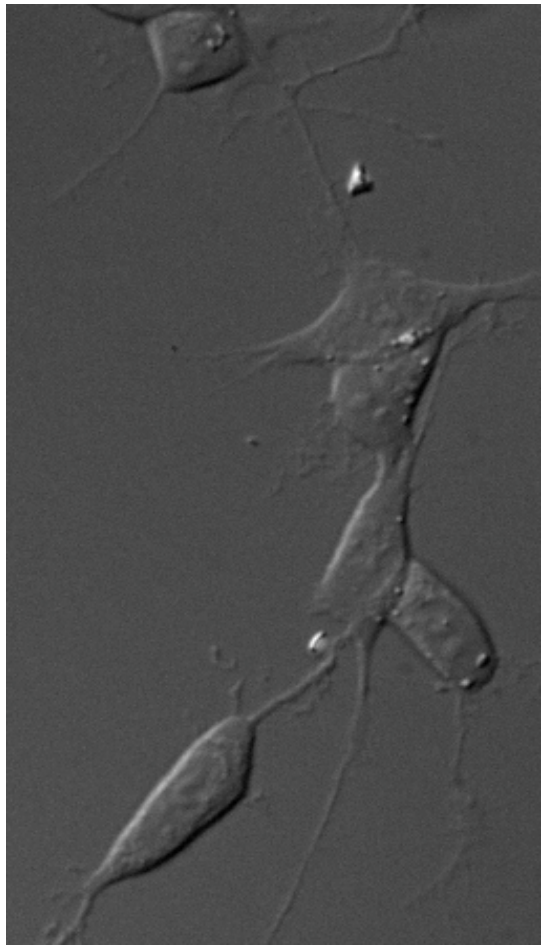
Molecular ProbesTM: Product Information, Fluo Calcium Indicators

Protocol continued

- Prep sample holders (vacuum grease)
- Transfer slides to holders and apply 950 µL HBSS
- Imaging: 488 nm…bf exposure 0.1 & gain 1… fluorescent exposure 0.5 & gain 100
- Adding Reagents: Aß42/Buffer ~ frame 95 … Ionomycin ~ frame 400 (50 µL both)
- Quantifying: Normalized by [Ca2+]free= [F-BG]/[F_i$-$BG$_i$]

Molecular ProbesTM: Product Information, Fluo Calcium Indicators

6/23/11 – SHSY5Y, Aß42



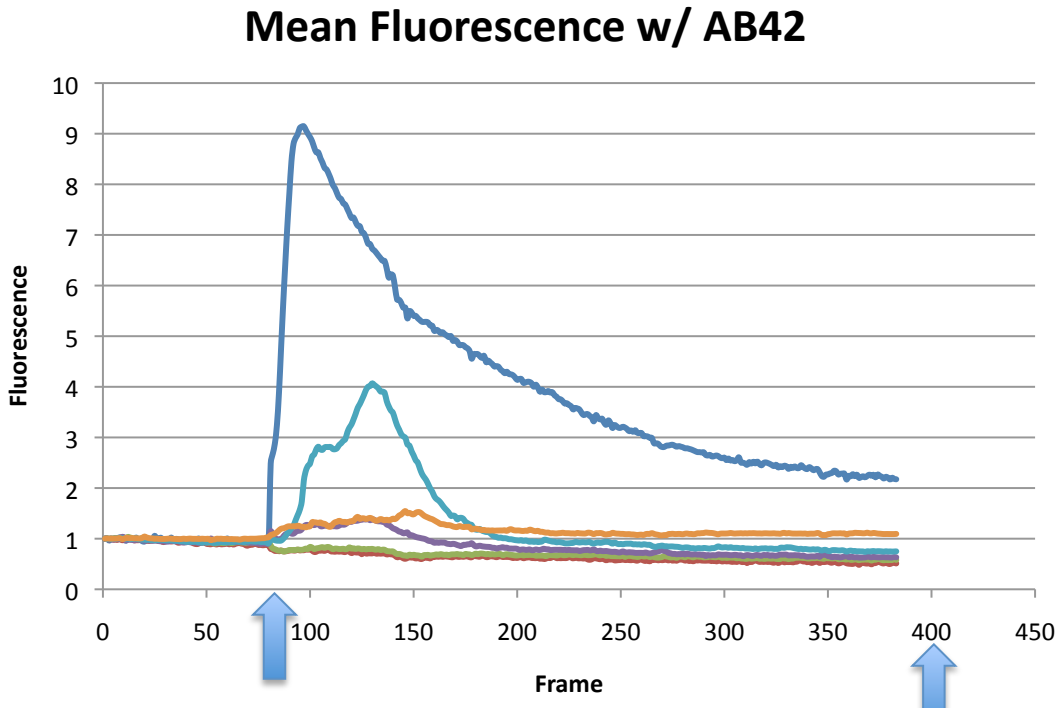
•Added Aß42 ~ frame 95

• No iono... evidently



6/23/11 – SHSY5Y, Aß42 – Mean Fluorescence

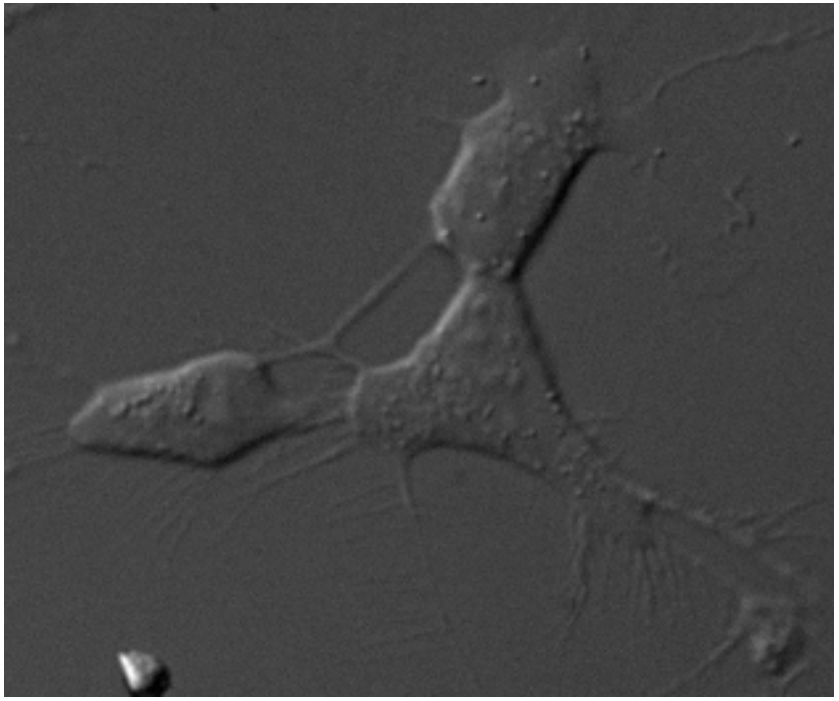


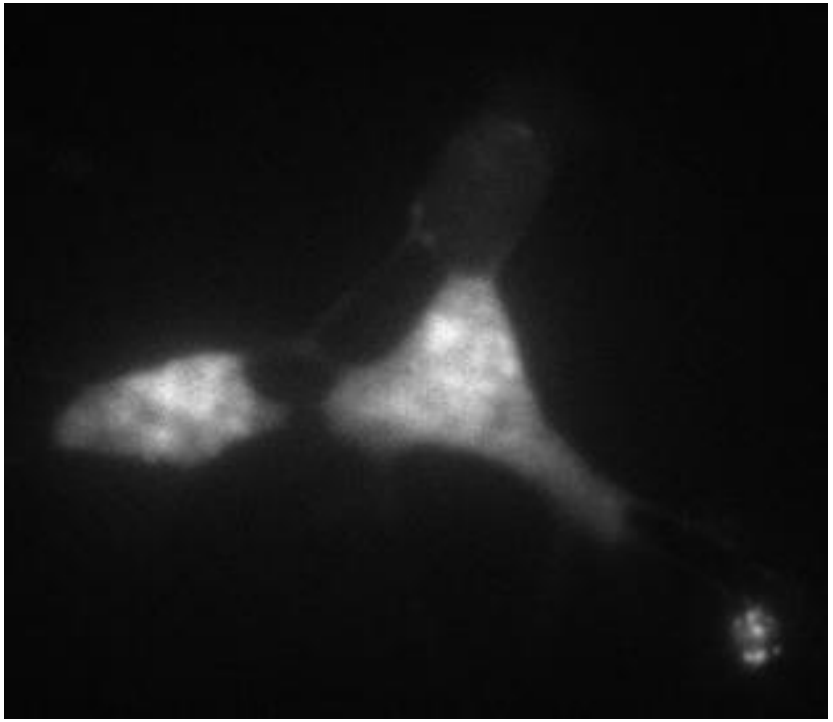


Aß42 ~ 95

Ionomycin ~ 400

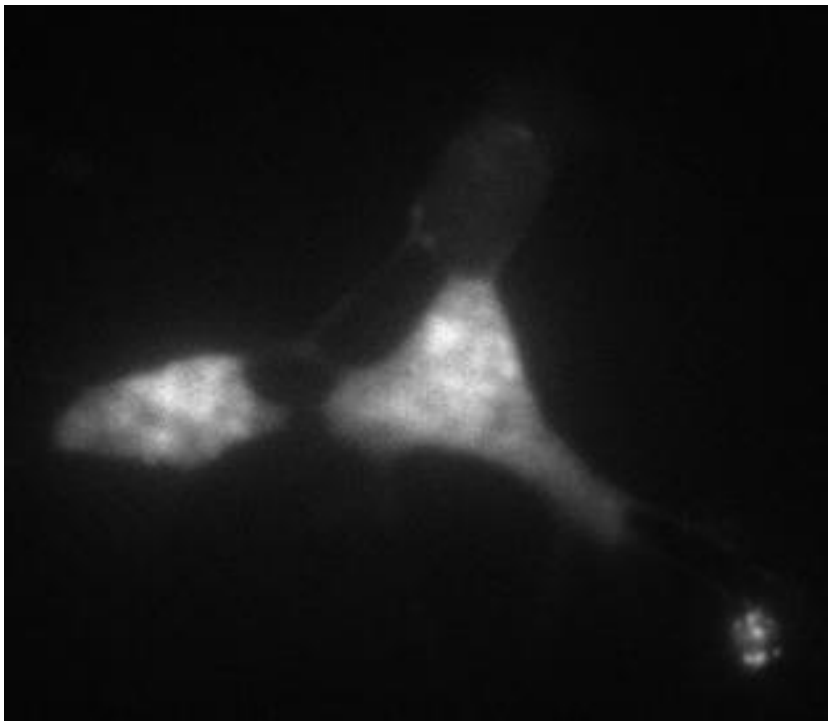
6/23/11 – SHSY5Y, Buffer

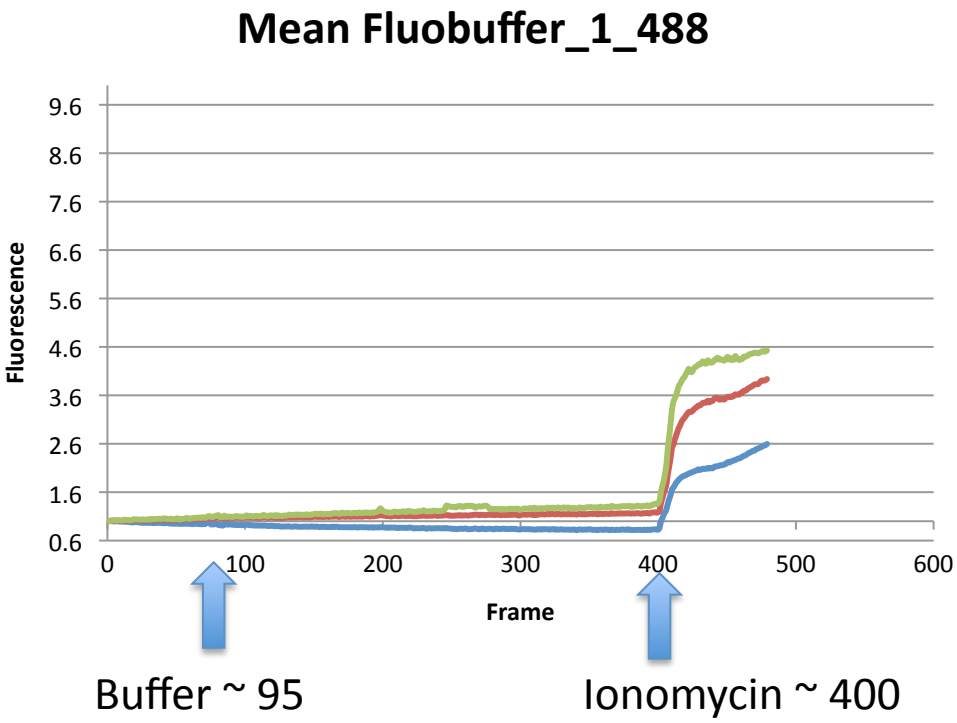




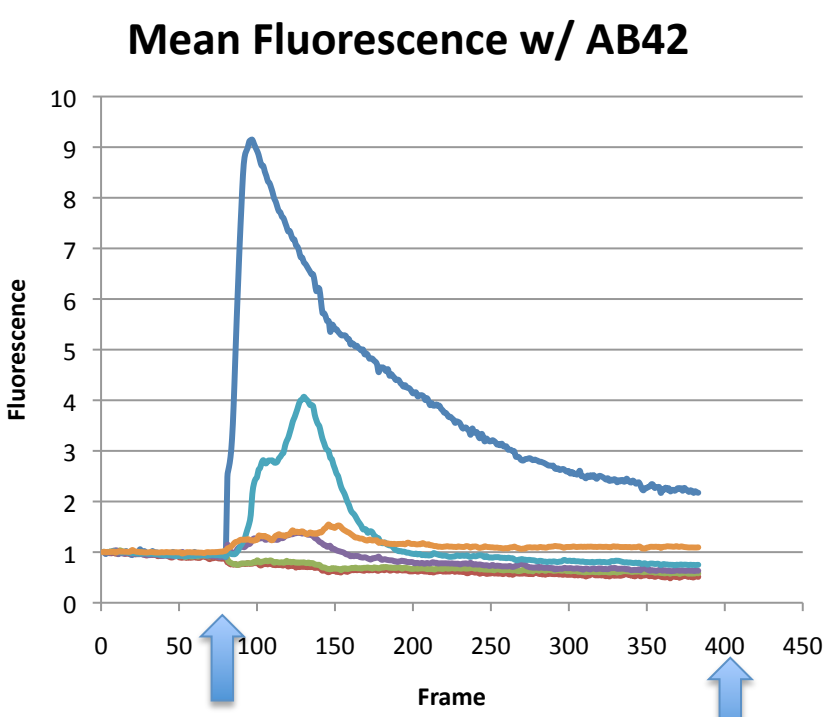
- Buffer ~ frame 95
- Ionomycin ~ frame 400

6/23/11 – SHSY5Y, Buffer – Mean Fluorescence



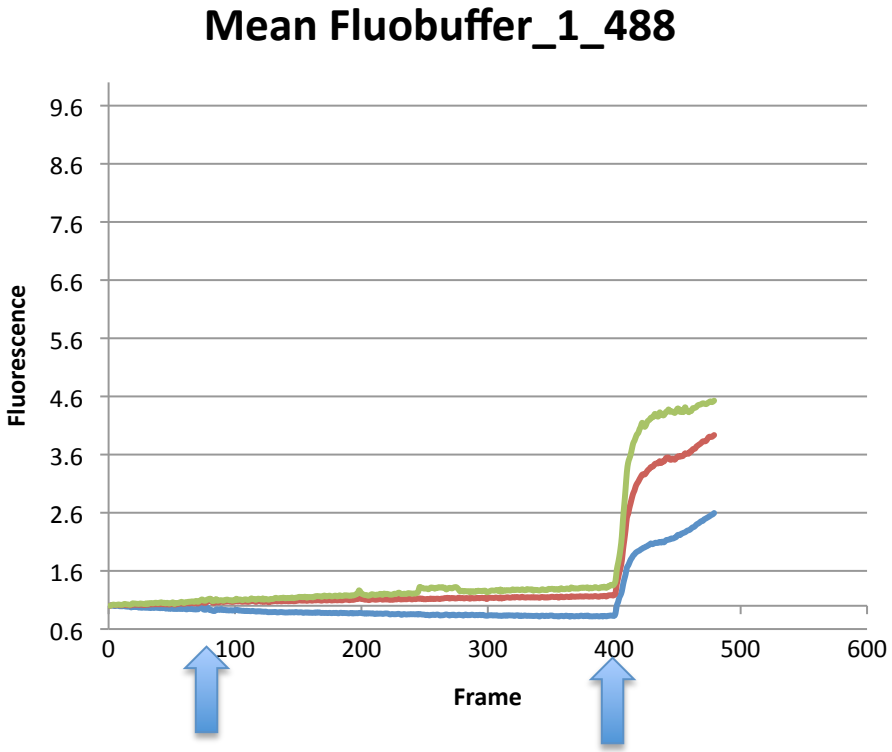


6/23/11 – Aß42, Buffer - Mean Fluorescence



Aß42 ~ 95

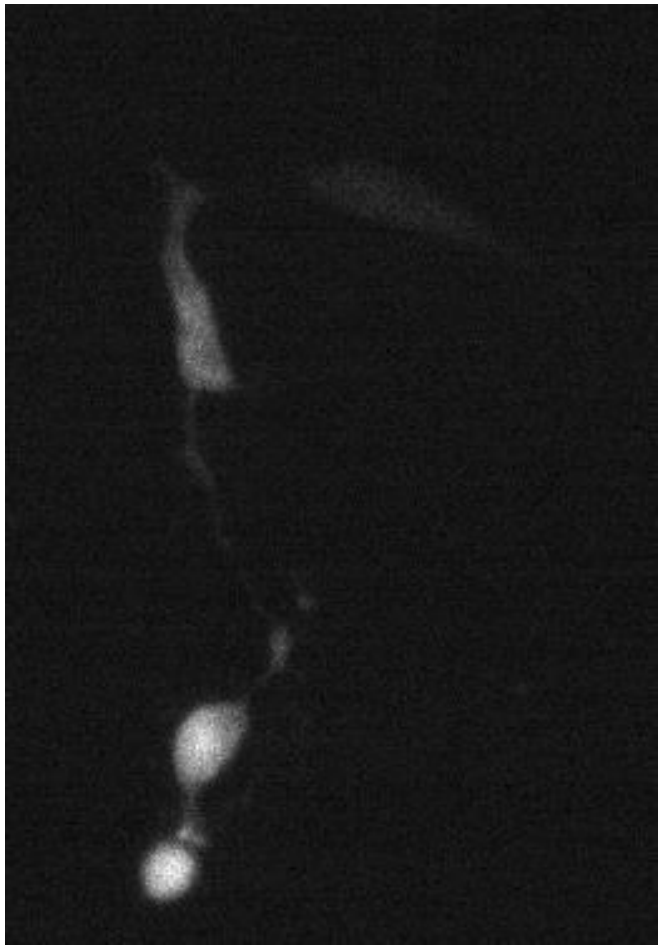
Ionomycin ~ 400



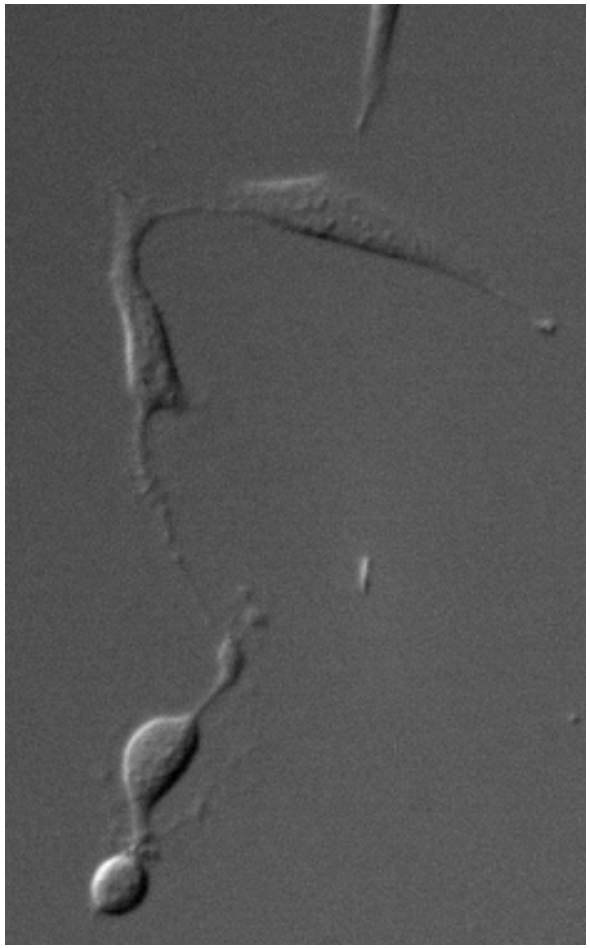
Buffer ~ 95

Ionomycin ~ 400

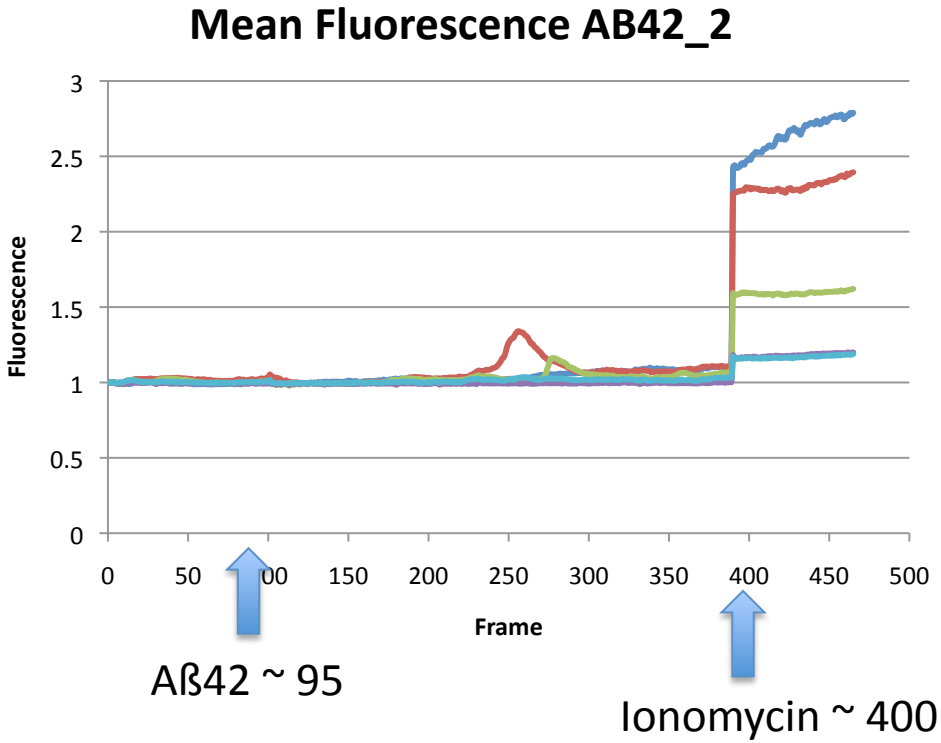
7/13/11 – SHSY5Y, Aß42

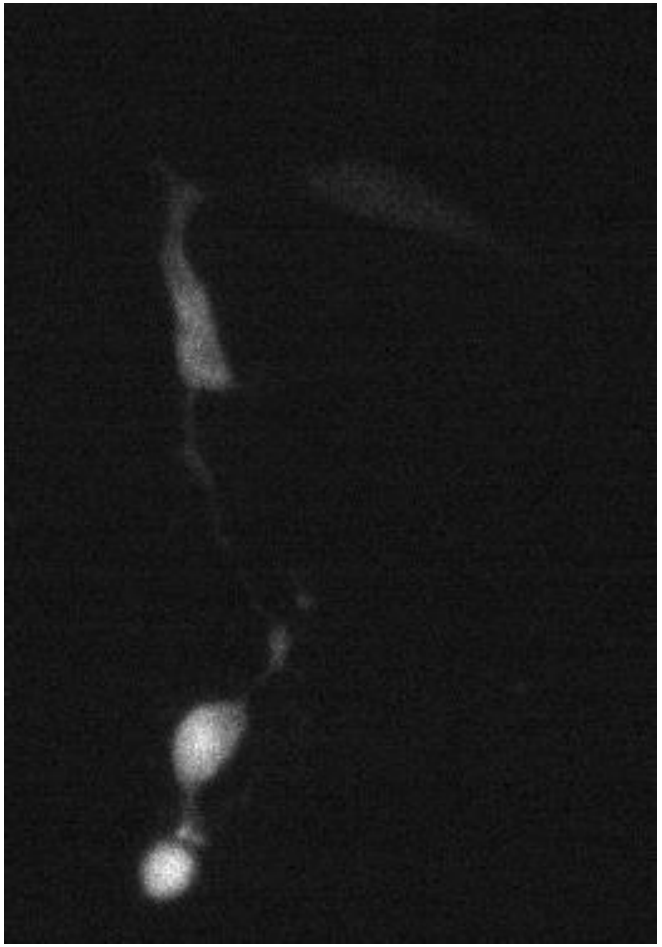


- Aß42 ~ frame 95
- Iono ~ frame 400

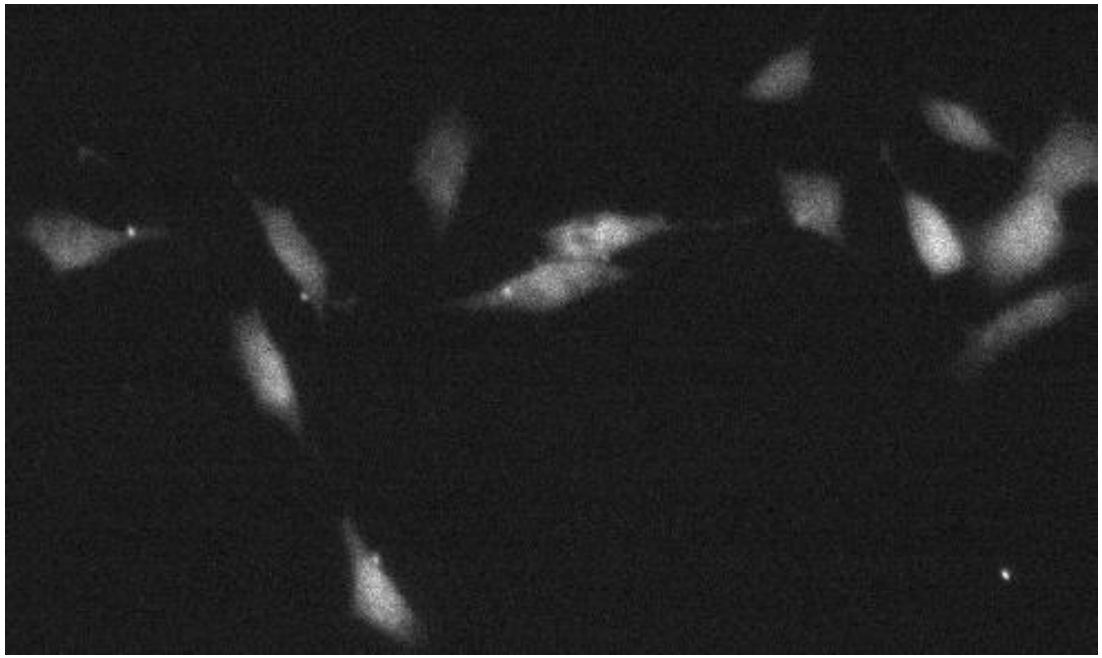


7/13/11 – Aß42 Mean Fluorescence



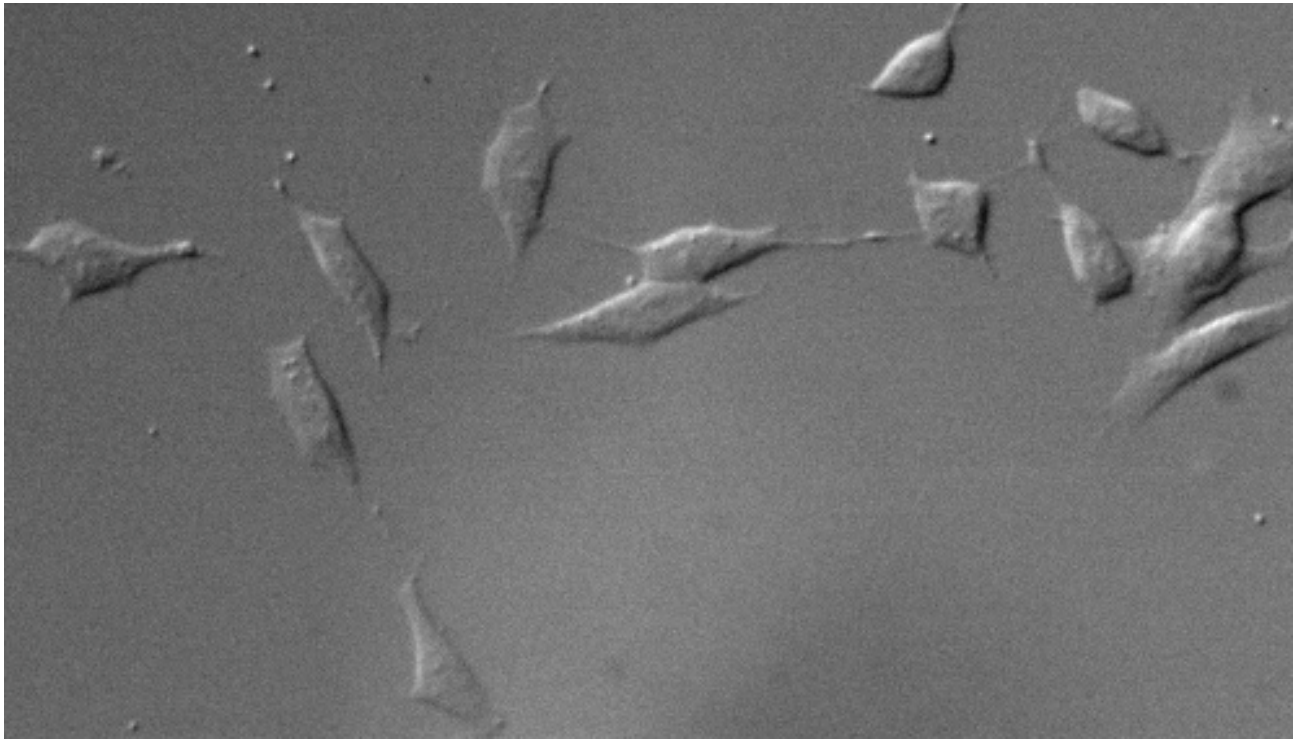


7/13/11 – SHSY5Y, Buffer

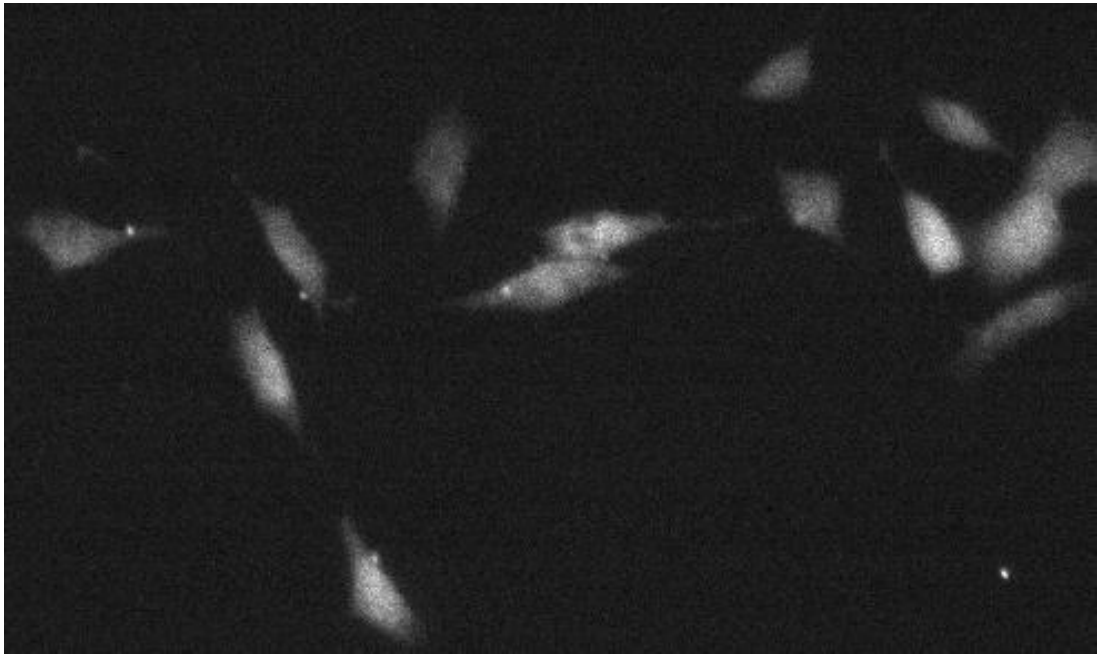


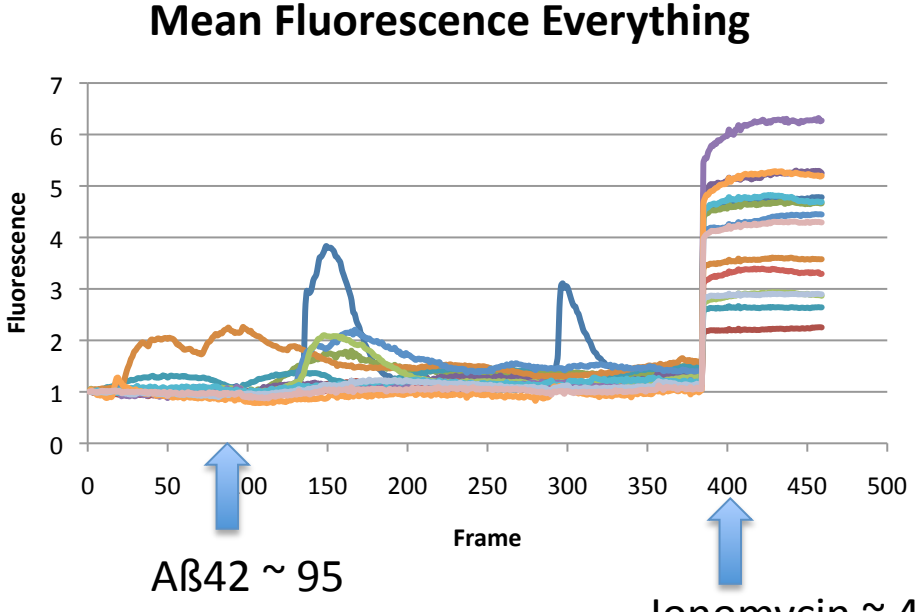
- Buffer ~ 95
- Ionomycin ~ 400

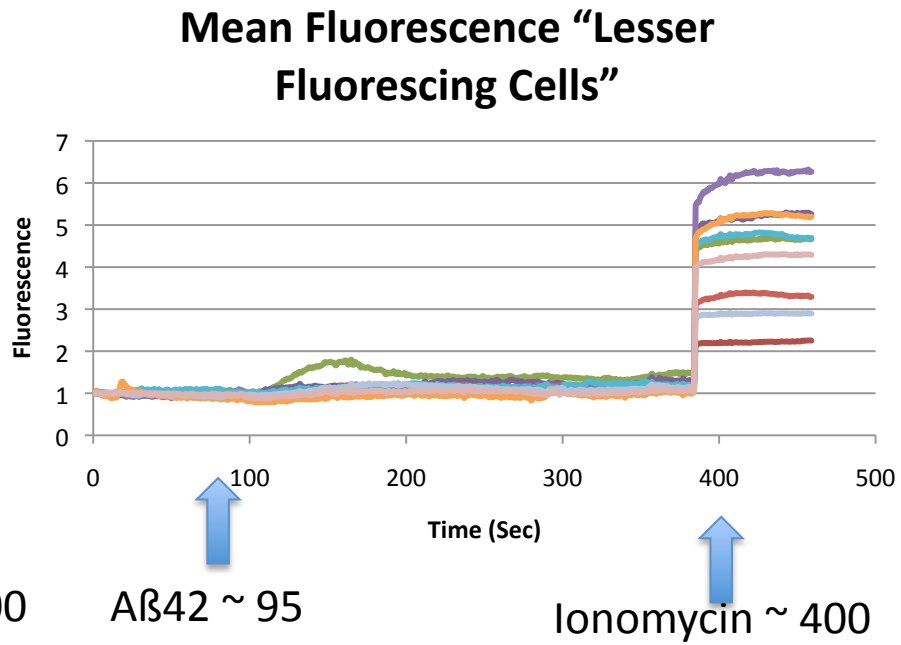
7/13/11 – SHSY5Y, Buffer… continued



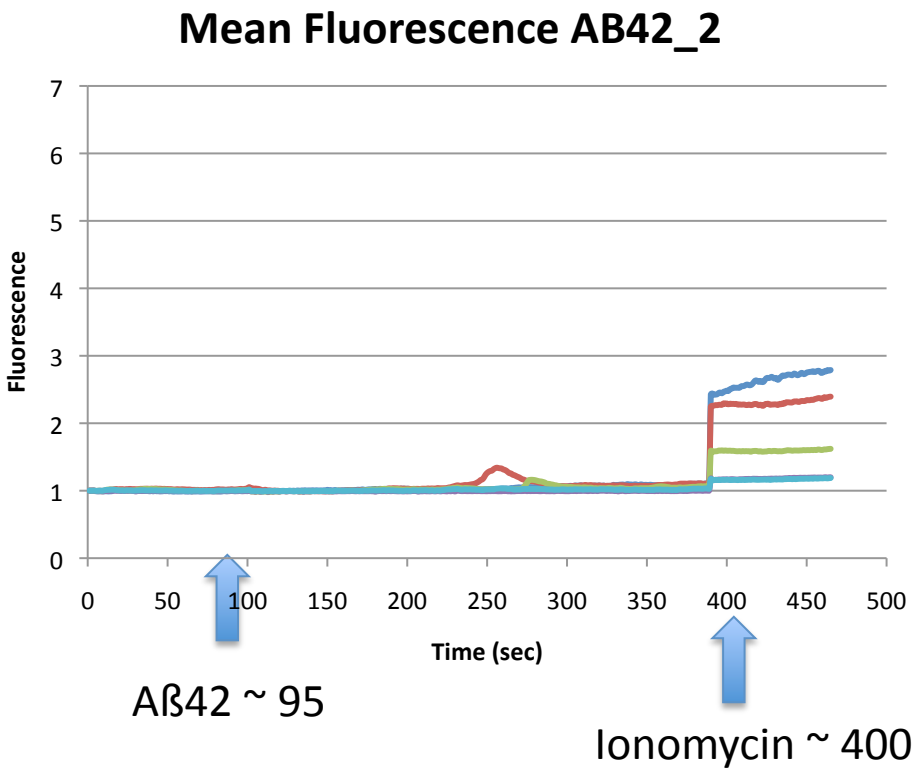
7/13/11 – Buffer Mean Fluorescence

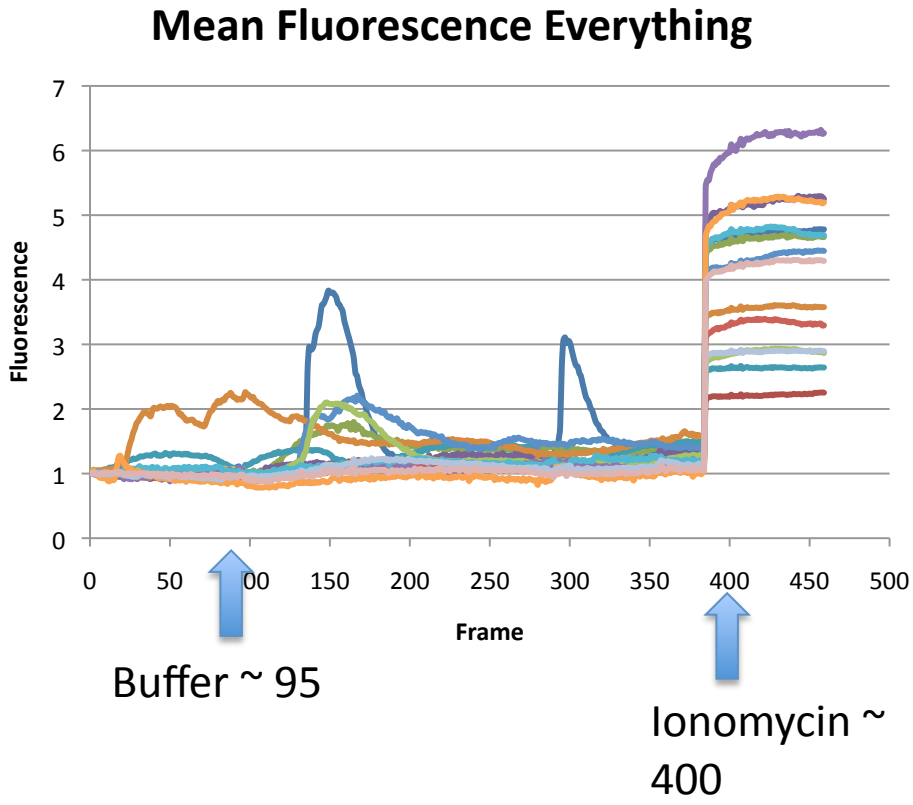






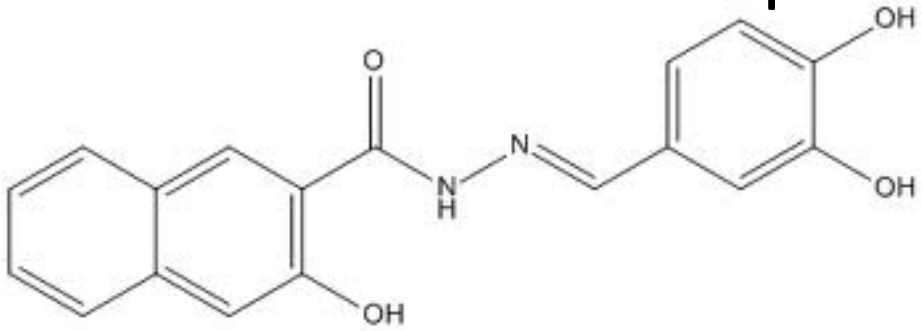
7/13/11 – Mean Fluorescence Comparison





7/26/11 – Implementing Dynasore

- Dynasore: Small molecule inhibitor of dynamin-guanosine triphosphate (GTP); blocking endocytosis (non-competing)
- Block lipid-binding, endocytosis to characterize whether it increases ability Aß to perforate membrane
- If doesn't take up, maybe continuing aggregation on membrane will perforate.



Biovision.com

Macia, E, Ehrlich M, Massol R, Brunner C, Kirchhausen T. (2006) Developmental Cell

7/26/11 – Protocol - Dynasore

- Aliquots:

  1) 40mL HBSS (washing)

  2) 10mL HBSS (Fluo4)

  3) 15mL HBSS (Dynasore)

→Water bath at 37°C for 15 min

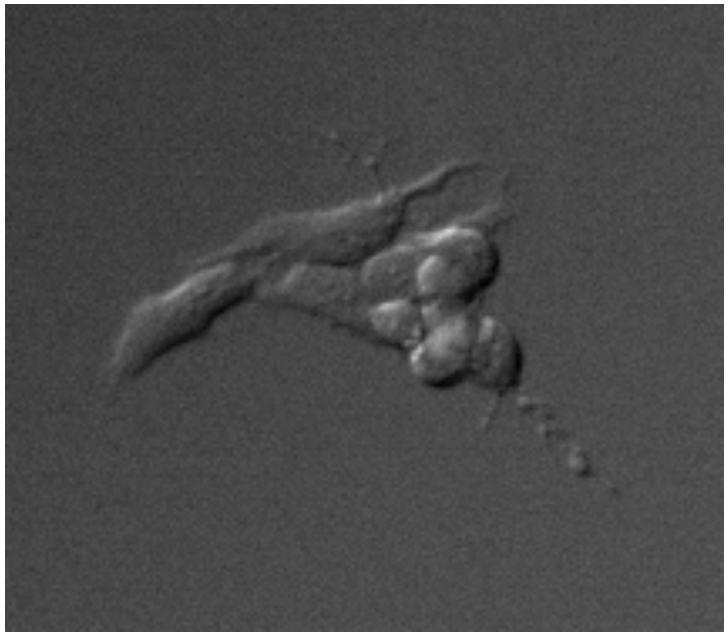
- Bring up Fluo4 (3µM), Aß42 (1µM), Buffer (500µL), Dynasore(80 µM, 0.2% DMSO), Melittin (4µM)
- 1) Incubate with ~ 2mL Dynasore for 30 min

  2) Incubate with ~ 2mL Fluo4 for 15 min

  3) Incubate again with ~ 2mL Dynasore 15 min

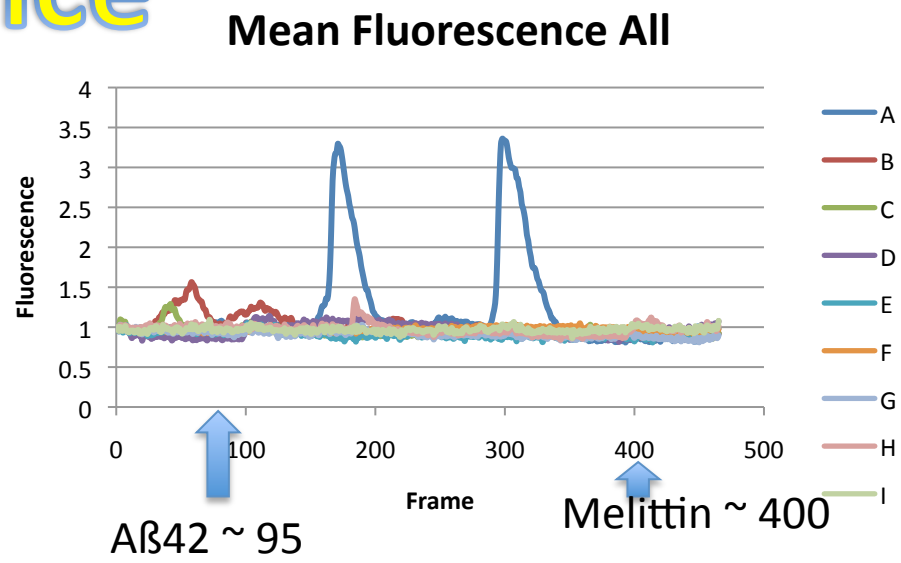
7/26/11 – SHSY5Y, Aß42 - Dynasore

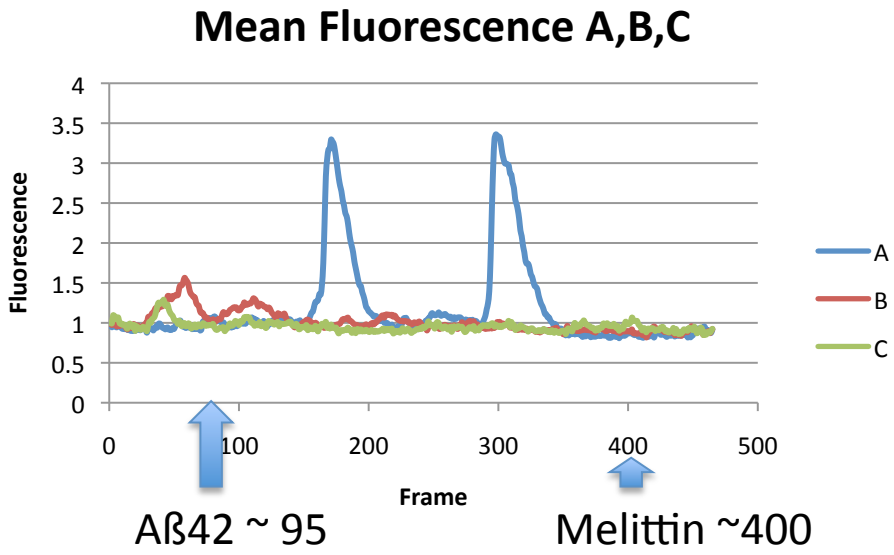




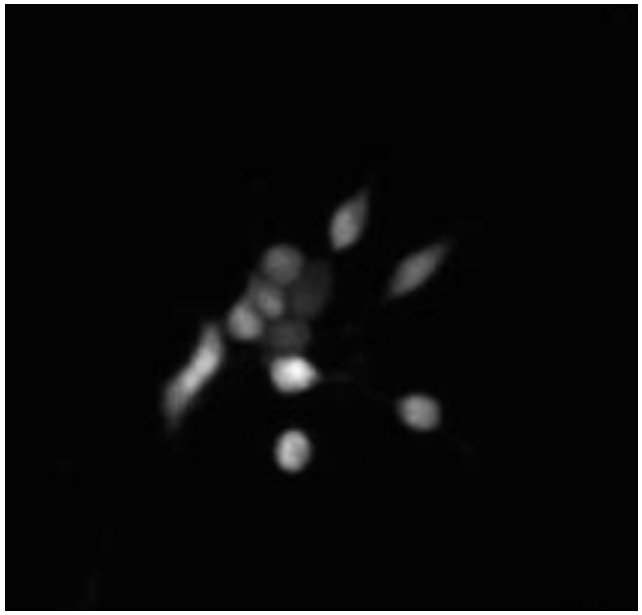
7/26/11 - Aß42 – Dynasore – Mean Fluorescence

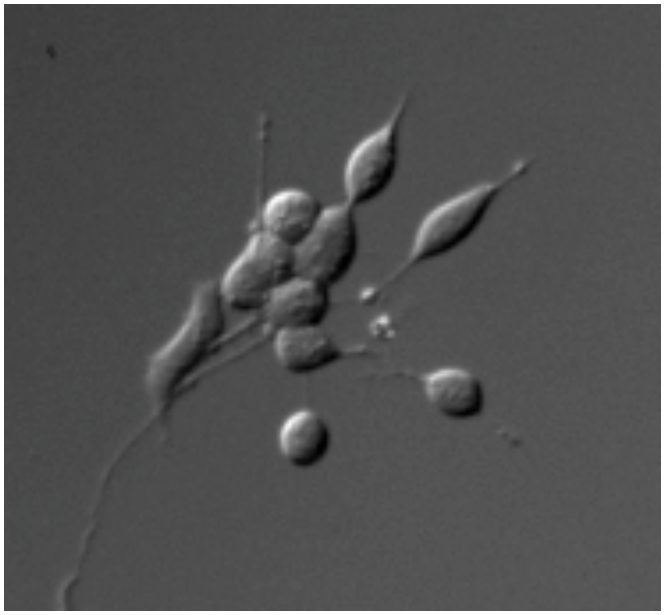




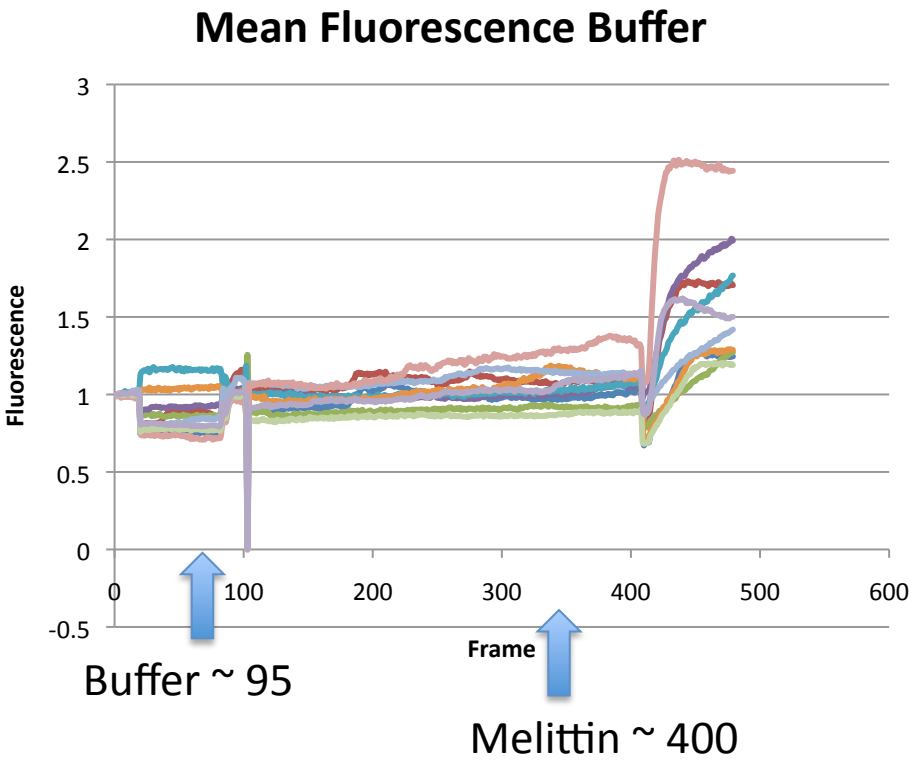


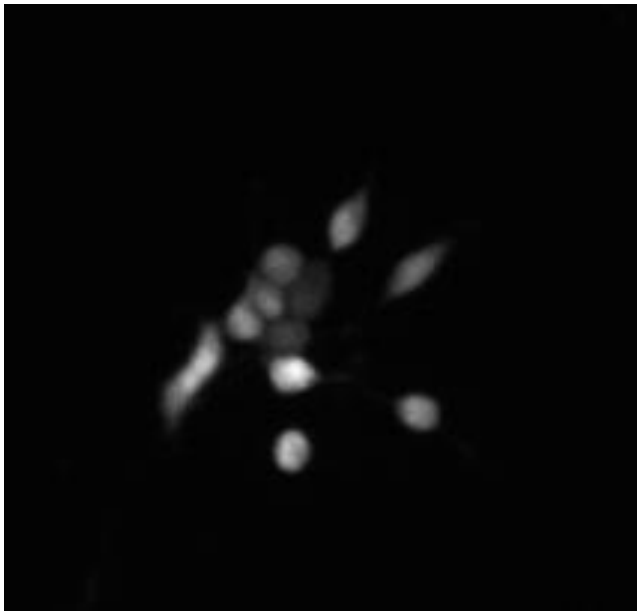
7/26/11 – SHSY5Y, Buffer - Dynasore



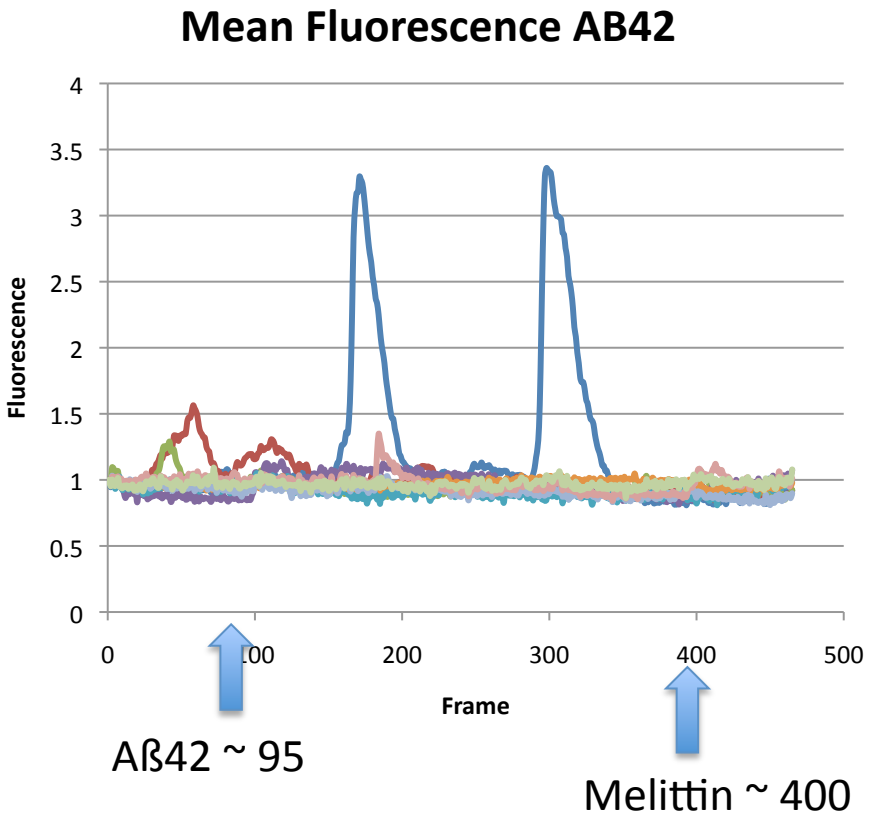


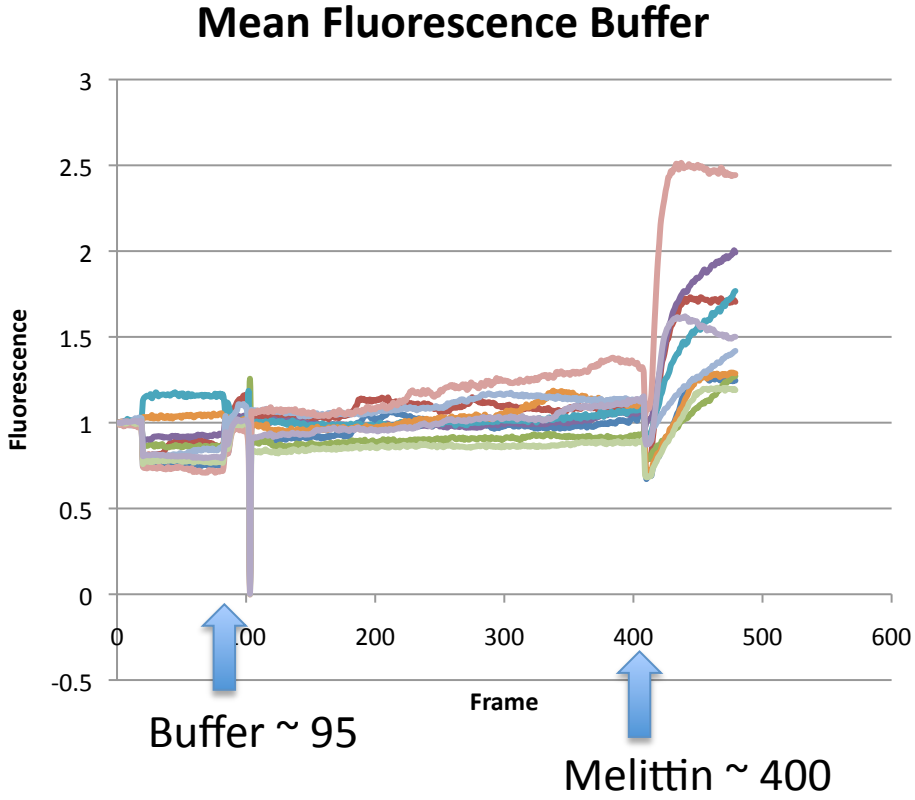
7/26/11 – Buffer, Dynasore – Mean Fluorescence





7/26/11 – AB42 & Buffer (Dynasore) – Mean Fluorescence



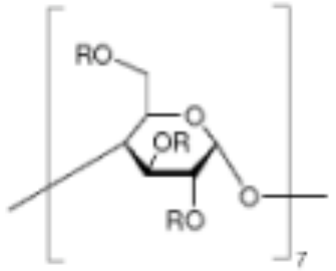


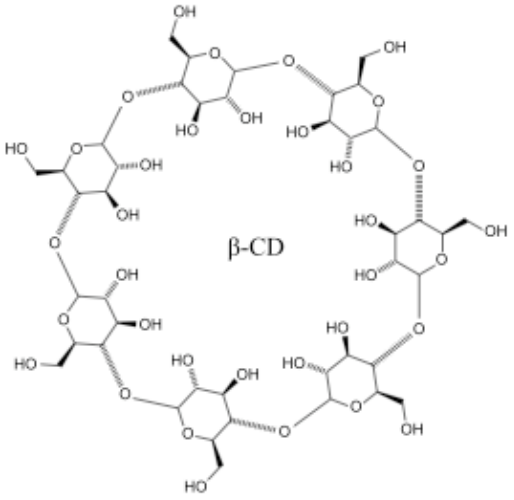
8/3/11 – Methyl ß cyclodextrin

- MßCD: Hydrophobic ring structure allows complex formation with other hydrophobic structures, i.e. cholesterols

  - Cholesterol extraction(1mM)

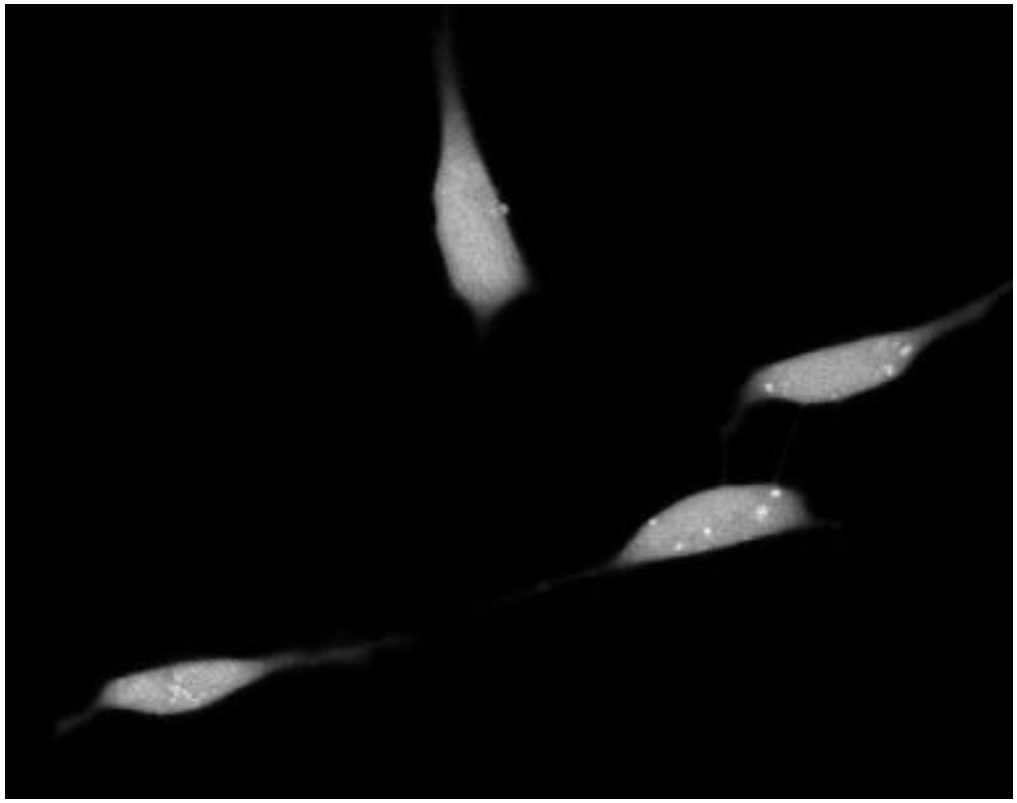
- Looking to increase extracellular ion exchange by weakening membrane
- Interfering with endocytosis and lipid rafts

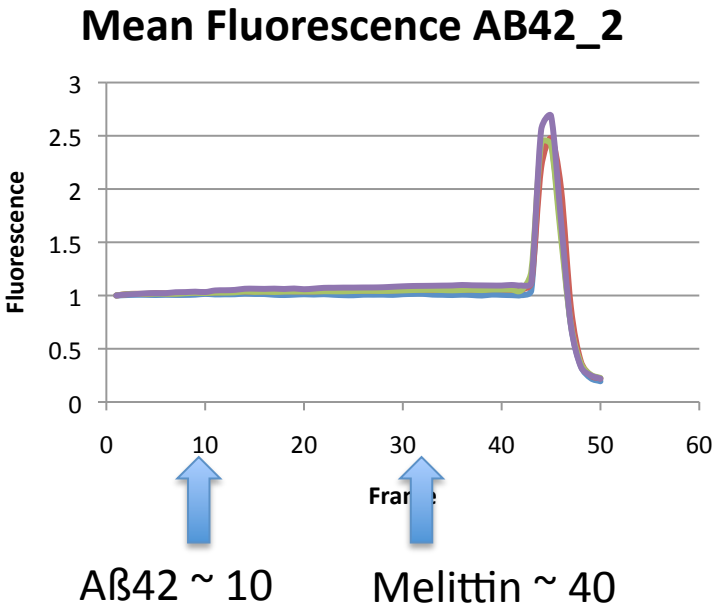




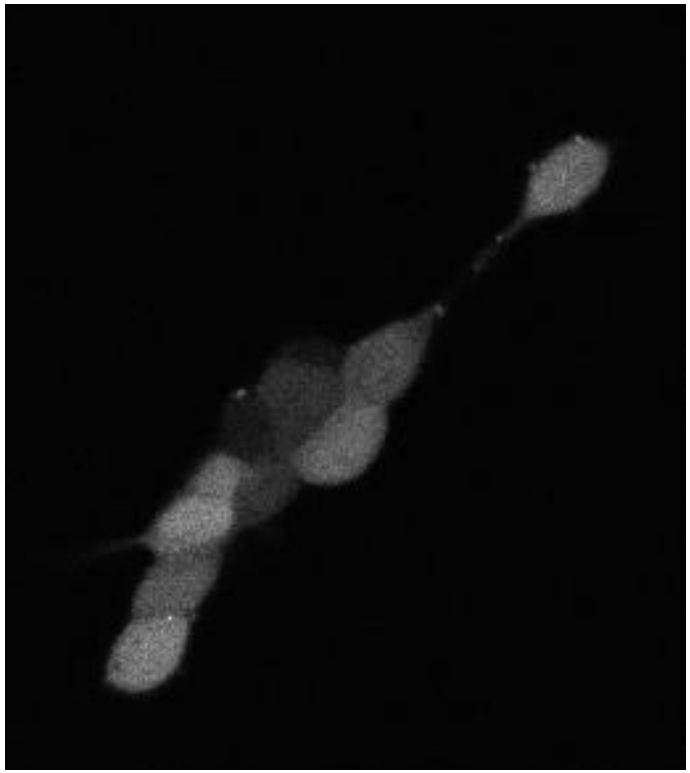
Depry, C, Allen, M.D, Zhang, J. (2010) RSC Publishing

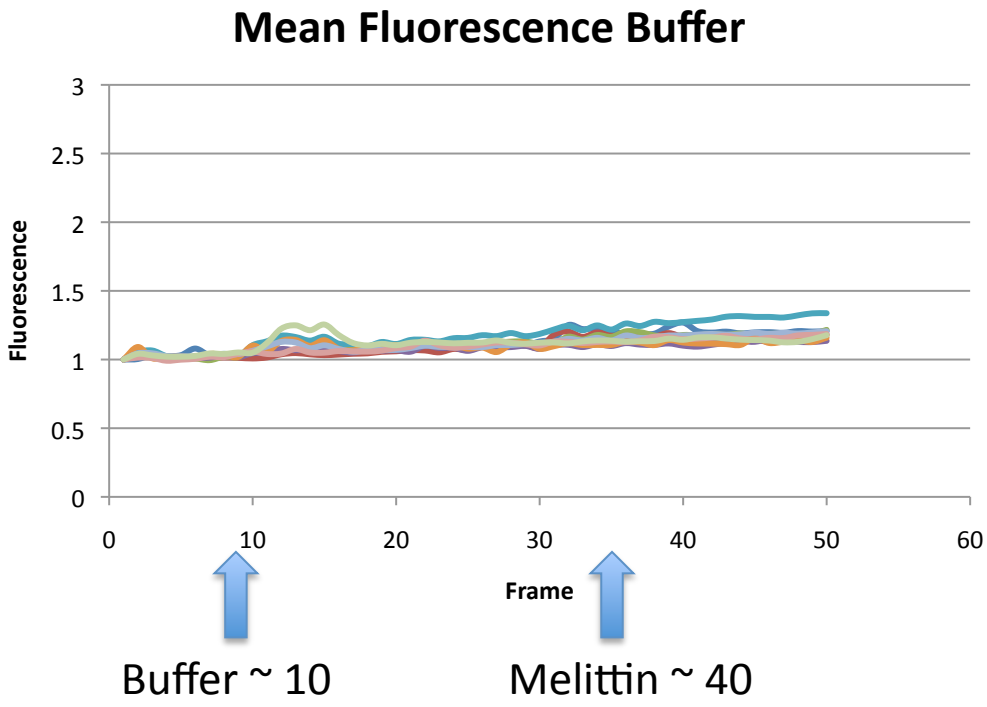
8/3/11 – AB42 + MßCD



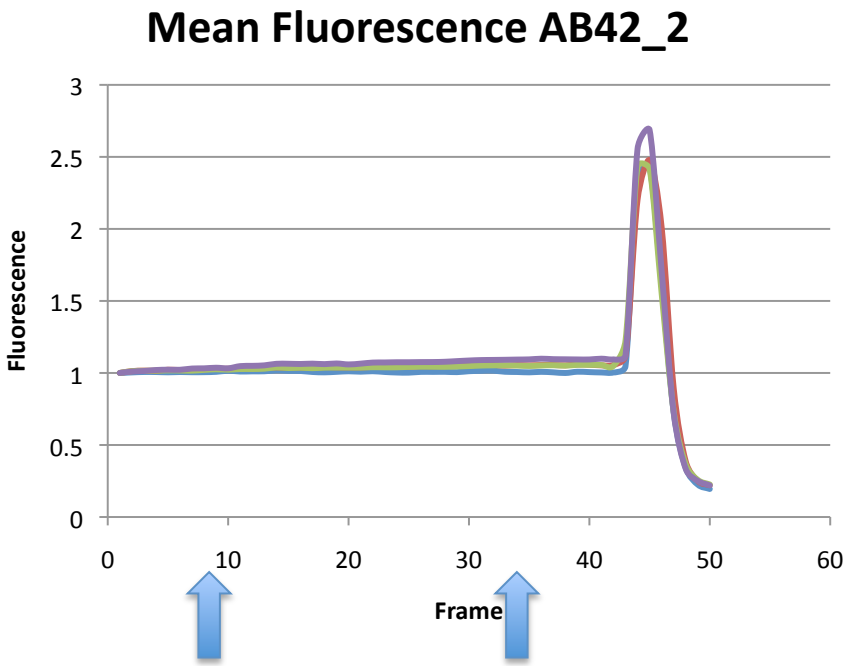


8/3/11 – Buffer + MßCD – Mean Fluorescence



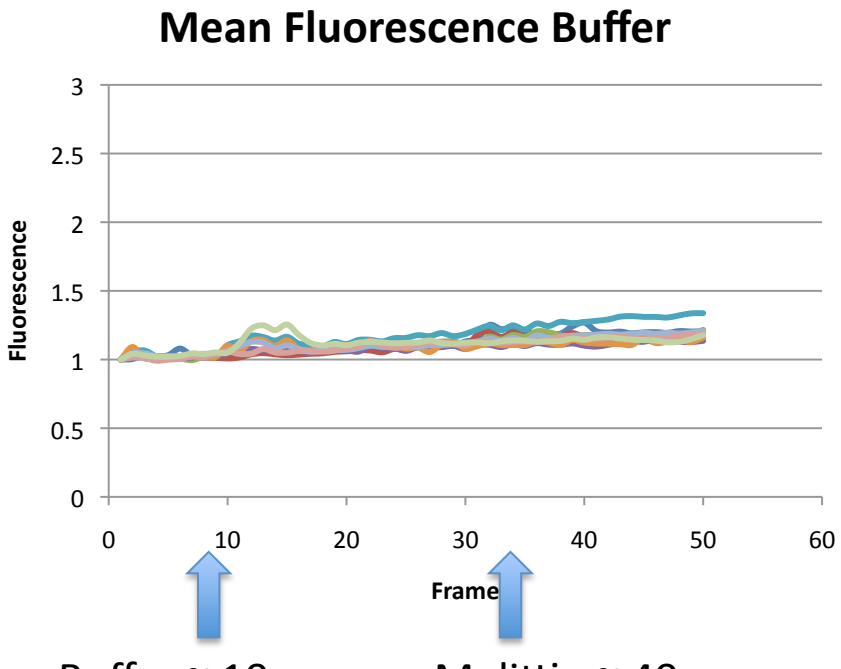


8/3/11 – AB42 & Buffer (MßCD) – Mean Fluorescence



Aß42 ~ 10

Melittin ~ 40



Buffer ~ 10

Melittin ~ 40

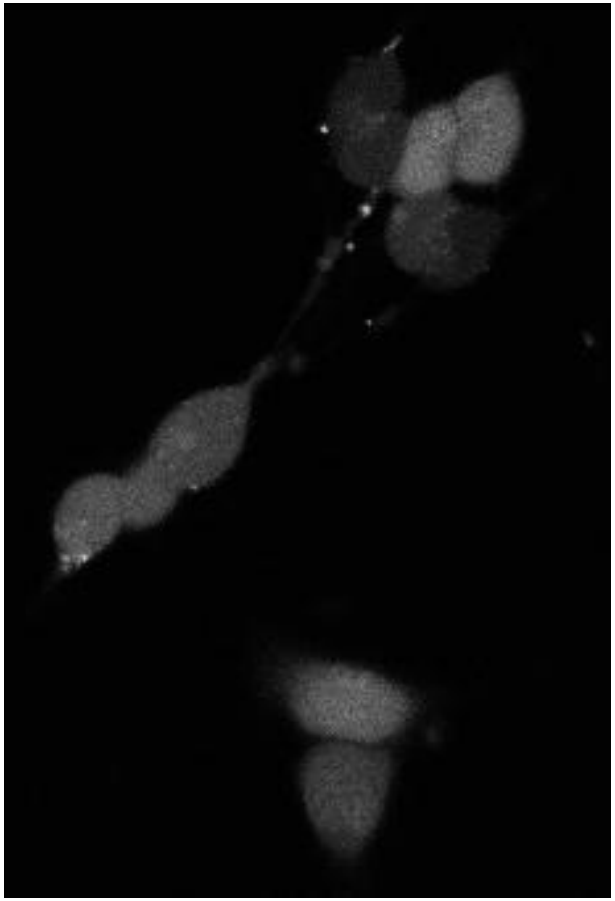
8/4/11 – Implementing Italian - AB42

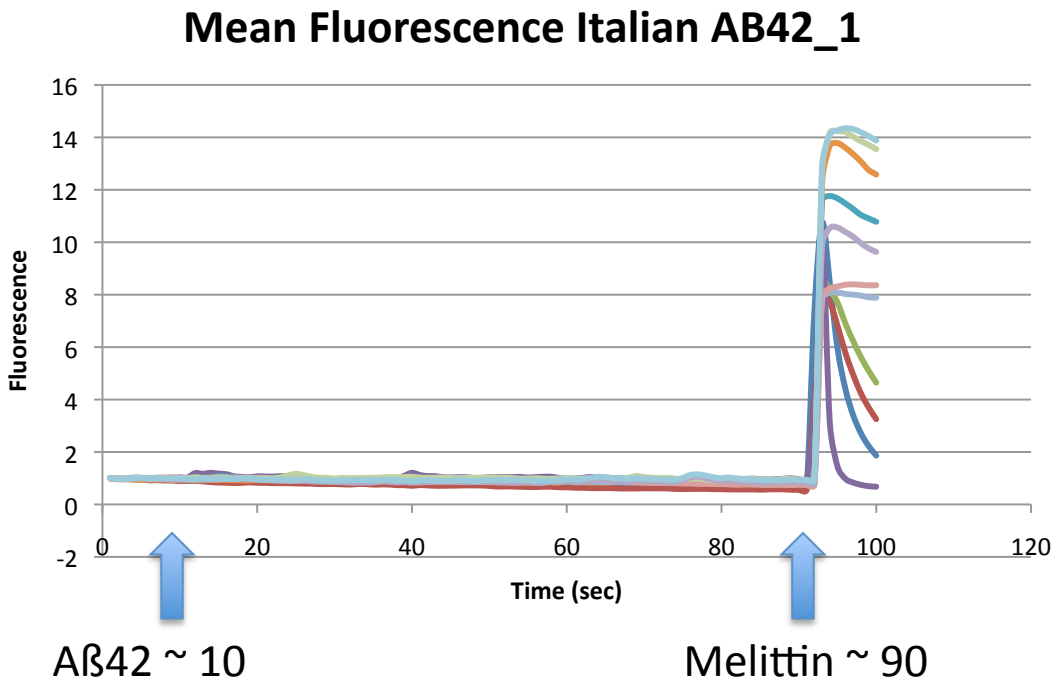
- Italian Mutation Aß42: Lysine substituted for glutamic acid at position 22. Aggregates more rapidly and is more toxic than wild-type Aß42.
- Hoping to see increased fluorescence, more cells responding, and calcium leakage.

H - Asp - Ala - Glu - Phe - Arg - His - Asp - Ser -
Gly - Tyr - Glu - Val - His - His - Gln - Lys - Leu -
Val - Phe - Phe - Ala - Lys - Asp - Val - Gly - Ser -
Asn - Lys - Gly - Ala - Ile - Ile - Gly - Leu - Met -
Val - Gly - Gly - Val - Val - OH

AnaSpec: Product Data Sheet [Lys22]-ß-Amyloid (1-42), Italian Mutation

8/4/11 – Italian:AB42 – Mean Fluorescence

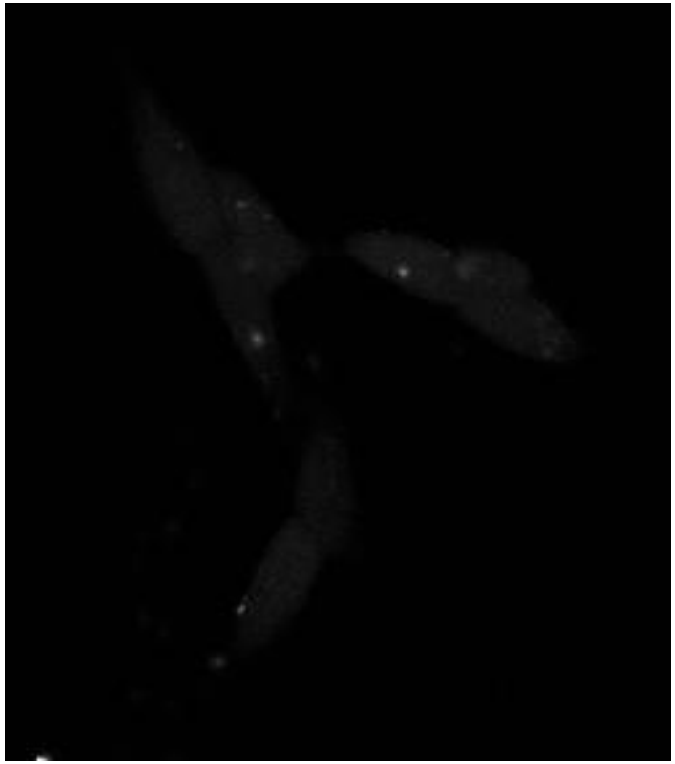


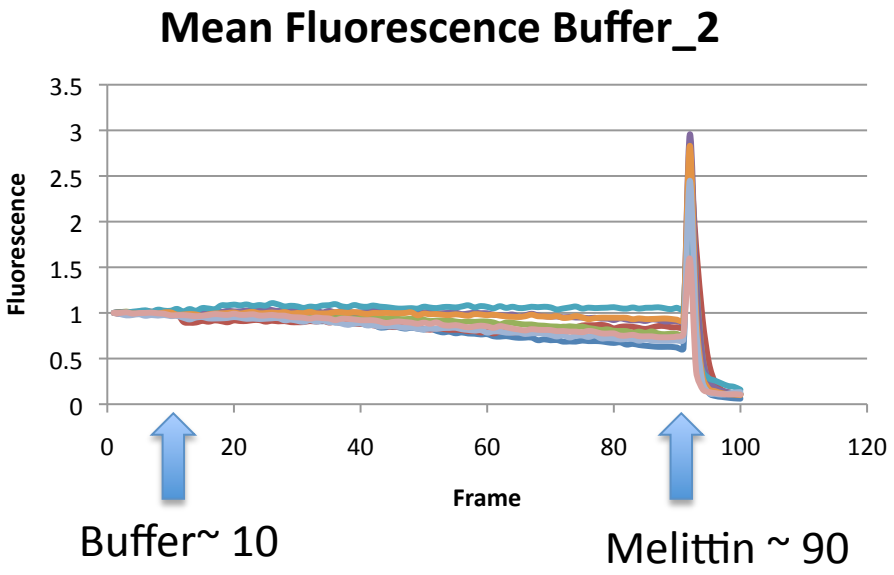


Aß42 ~ 10

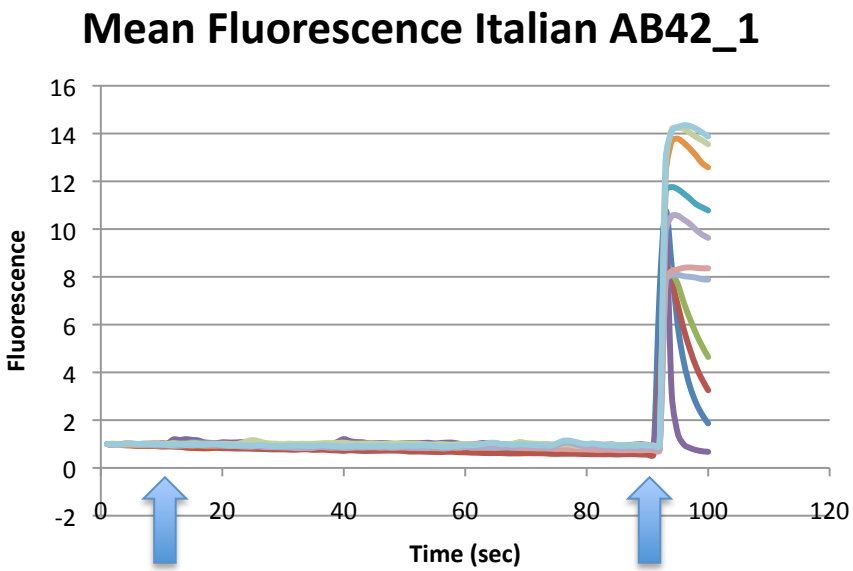
Melittin ~ 90

8/4/11 – Italian:Buffer – Mean Fluorescence



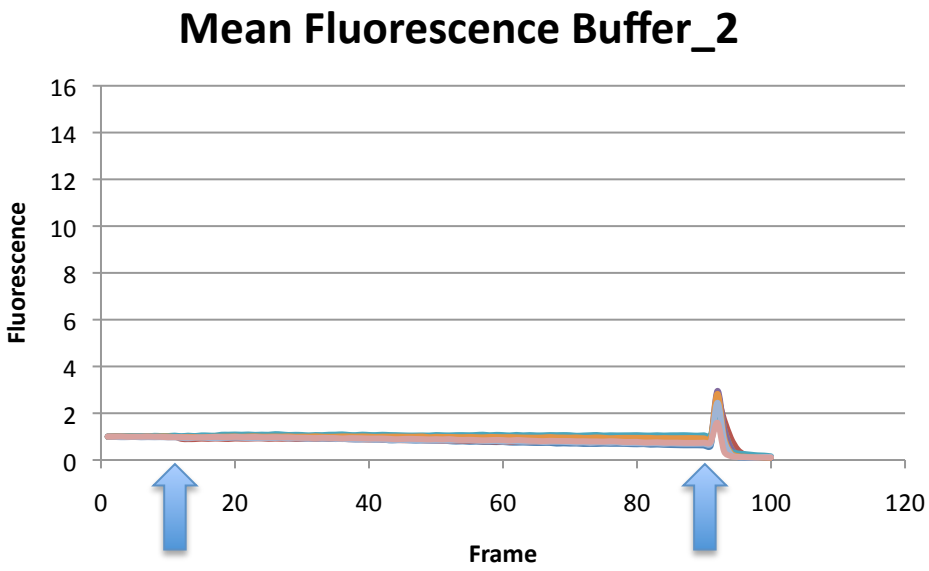


8/4/11 – Italian: Mean Fluorescence Comparison



Aß42 ~ 10

Melittin ~ 90



Buffer ~ 10

Melittin ~ 90

Conclusions

- Outdated cell media led to unhappy cells. Thus causing odd perforations and leaking (Italian)
- Ways to better the technique in the future...

  - Regular Aß42 Ca2+ indicator: Trying different laser and camera settings, like recording times… maybe try shorter videos or longer ones?

  - Ca2+ indicator+MßCD: 1mM might have been too weak. So try higher concentration. Also maybe try differently timed video segments and settings. (Wash off to reduce binding to Aß?)

Conclusions continued

- Ca2+ indicator+Dynasore: Trouble going into solution… might be dissolved better by lowering concentration slightly. Also, try with iItalian also?. Faster aggregation might show interesting fluorescence.
- Ca2+ indicator Italian: Trying different laser and camera settings, like recording times… maybe try shorter videos or longer ones.

Materials

- SHSY5Y – Human Derived Nueroblastoma
- Cell Media: DMEM:F-12 Phenol Red Free (stored 4^{o}C) : 10% Fetal Bovine Serum (stored -20^{o}C) : 200 units/mL Pen/Strep (10k units/mL, 1mL/50mL media) (stored -20^{o}C)
- Unlabeled ß-Amyloid (1-42): (3&8 µg)
- Italian ß- Amyloid(1-42): (5 µg)(Mutation)
- Fluo-4 AM (Acetoxymethyl): Brought up in DMSO
- Ionomycin: (Ionophore) Pore forming
- Melittin: Pore forming peptide.
- HBSS Buffer:
- Sodium Phosphate Buffer: pH 7.4 (NO salt)
- Dynasore: Dynamin endocytosis blocking (0.2% DMSO)
- Methyl-ß-Cyclodextrin: Cholesterol Extraction

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