8-8-2013

Unraveling the Mechanism of 2,4-Dinitrofluorobenzene-Associated Cell Death

Franklin Thelmo
Saint Joseph's University, sju.thelmo@yahoo.com

Unraveling the Mechanism of DNP-mediated Cell Death

Frankie Thelmo
Summer Training Program in Cancer Immunotherapy Intern
Thomas Jefferson University

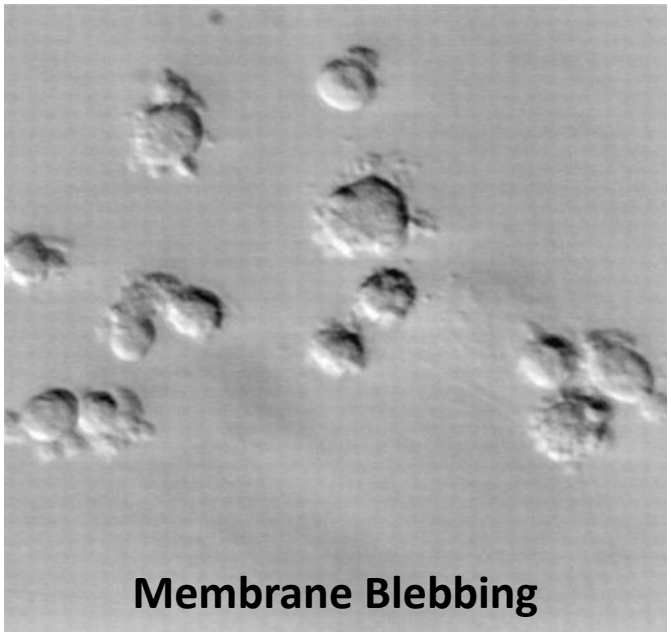
Types of Cell Death

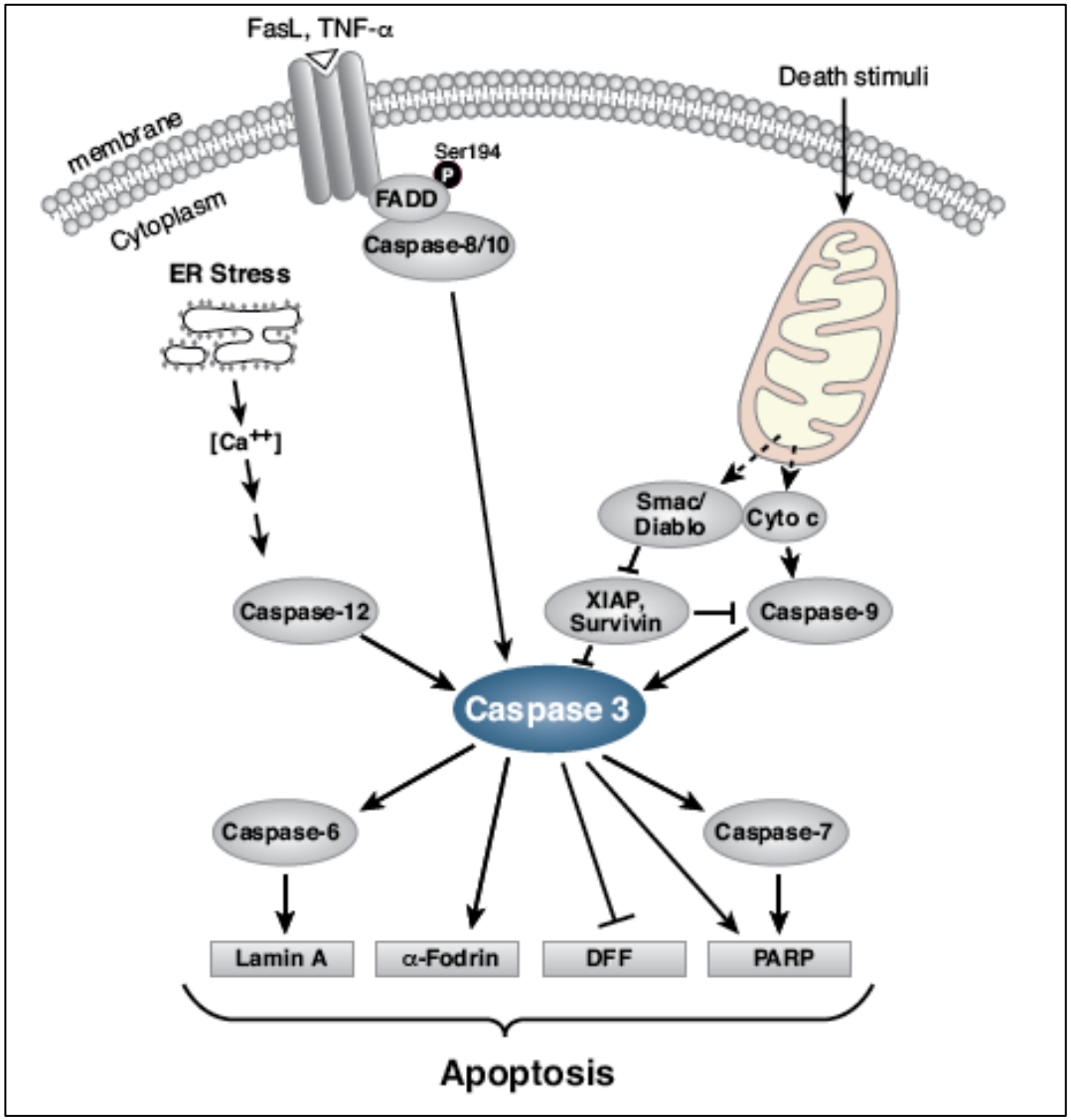
- Apoptosis: Caspase-dependent
- Necrosis: Physical Trauma
- Autophagy: Lysosomal digestion
- Pyrotopsis: Caspase-1 activation
- Paratopsis: Mitogen Activated Protein (MAP) kinase regulated
- Pyronecrosis: IL-1B and HMGB1 release

Kroemer, G., et al. *Cell Death Differ*. 2009. 16 (1): 3-11.

Apoptosis

- **Programmed cell death**
  - Development
  - Cell turnover
- **Typical appearances**
  - Phosphatidyl serine flipping
    - Annexin V staining
  - Apoptotic bodies and membrane blebbing
- **Staurosporine treatments**

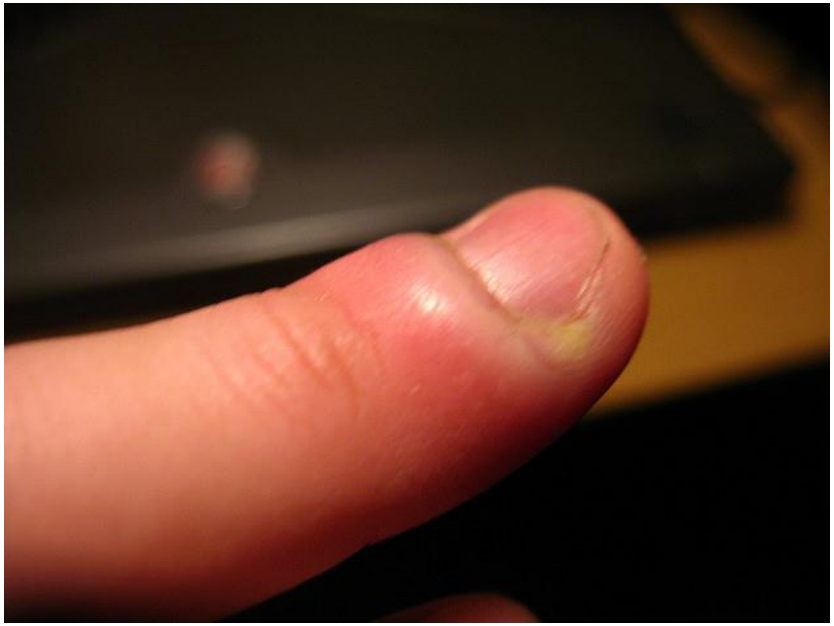




1. http://openi.nlm.nih.gov/detailedresult.php?img=2132458_JCB.
2. Cell Signaling, 9930S Apoptosis Kit Data Sheet

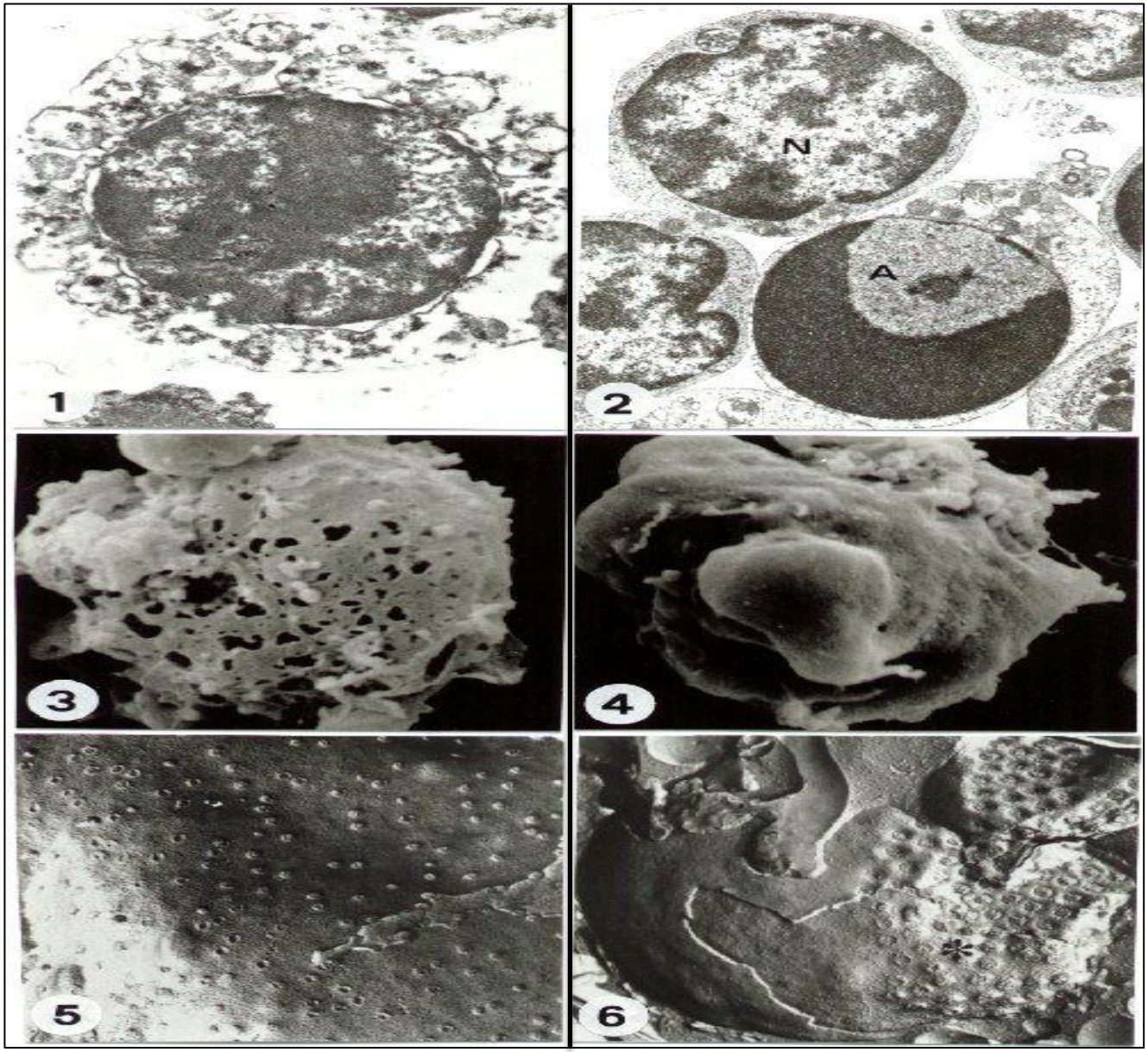
Necrosis

- Unregulated cell death
- Result of physical trauma to cell
- Release of cellular contents
  - Inflammatory Response
- Biomarkers released during Necrosis
  - Cyclophilin A
    - Isomerase
  - HMGB1
    - Transcription Regulator



http://www.flickr.com/photos/26953774@N02/3079248576/

Necrosis Apoptosis



http://www.cyto.purdue.edu/archive/flowcyt/research/cytotech/apopto/data/chap10.htm

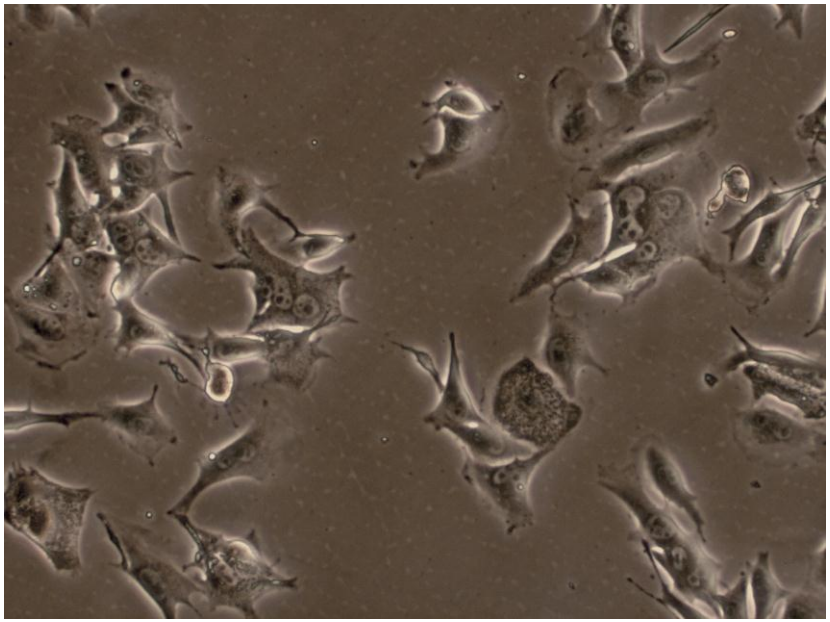
Hapten-mediated cell death

- Hapten = a small molecule that can elicit an immune response when bound to a carrier protein
  - The lab uses the hapten 2,4-Dinitrofluourbenzene (DNFB), which is known as dinitrophenyl (DNP) when bound to a carrier protein or cell
- Fujiwara et al, 1984 created a hapten mediated tumor regression model in mice
- Clinical trials by Berd et al, 1993-2004, used a DNP-modified autologous melanoma vaccine to treat stage III and IV melanoma patients → 45% 5 yr overall survival and immune correlations
- DNFB (low concentrations) is capable of reducing cellular metabolism in an MTT assay (Cruz et al, 2003)
  - Annexin/PI flow cytometry for apoptosis
  - Caspase-3 cleavage
- DNFB (low concentrations) treated cells are apoptotic at low concentrations (Manome et al, 1999)
  - Annexin/PI flow cytometry for apoptosis
  - Microscopy
- These papers and preliminary lab research suggests necrosis at high DNFB concentrations

1. http://www.fishersci.com/ecomm/servlet/fsproductdetail_10652_10398682__-1_0
2. Cruz, M.T. et al. *International Journal of Toxicology*. 2003. (22):43-48.
3. Manome, et al. *Immunology*. 1999. 98(4): 481-490.

B16F10 Melanoma Cell Line

- First established in 1973 by I. J. Fidler.
- B16F10 cells are an established mouse melanoma cell line. Used extensively for melanoma research.
- B16F10 is a syngeneic mouse melanoma cell line which causes tumor formation and metastasis in C57BL/6 mice.



Fidler, I.J. : Nature (London) New Biol., 242 : 148-149, 1973

DNP-mediated Melanoma Vaccine

- We are currently investigating a mouse model for a *in situ* DNP-mediated vaccine for metastatic melanoma

- DNP-mediated cell death mechanism of the tumors will be important to understand how our *in situ* vaccine elicits anti-tumor immunity

Green D.R., et al. *Nat Rev Immunol*. 2009. 9 (5): 353.
Melcher, A., et al. *Journal of Molecular Medicine.* 1999. (77): 824-833.

Purpose

2,4-dinitro-1-fluorobenzene (DNFB) is a strong hapten that can cause cell death, the mechanism by which is unclear.

We will modify B16F10 cells with 0.52mM for various time points and then stain with apoptotic and necrotic specific markers.

These results could help us understand how DNFB is affecting B16F10 Tumors in the lab's DNP-mediated tumor regression model.

Previous Lab Research

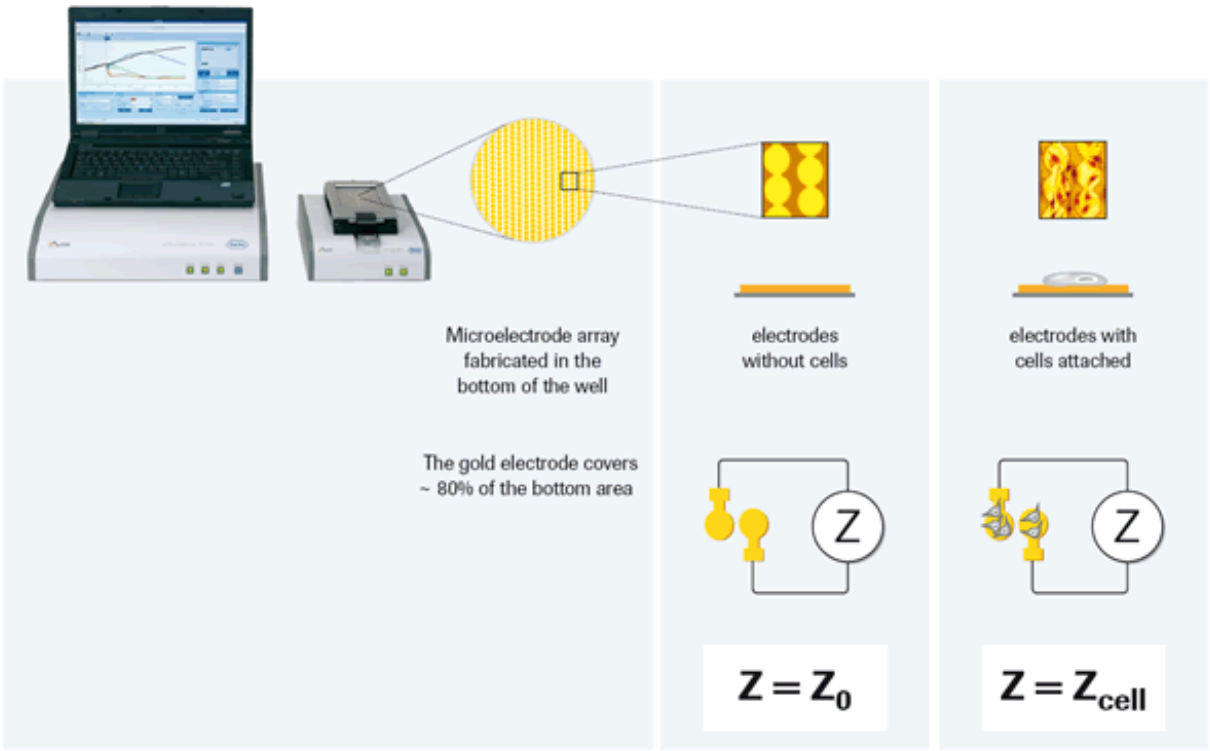
DNP-mediated Cell Death

- The purpose is to evaluate the general mechanism of cell death associated with DNP-modification
- Currently thought to be cytolytic via membrane permeabilization
- The lab has used Annexin V and 7-AAD as well as Caspase-3 to show cell death
  - Used Staurosporine as a positive control for apoptosis

Dan Erkes, unpublished

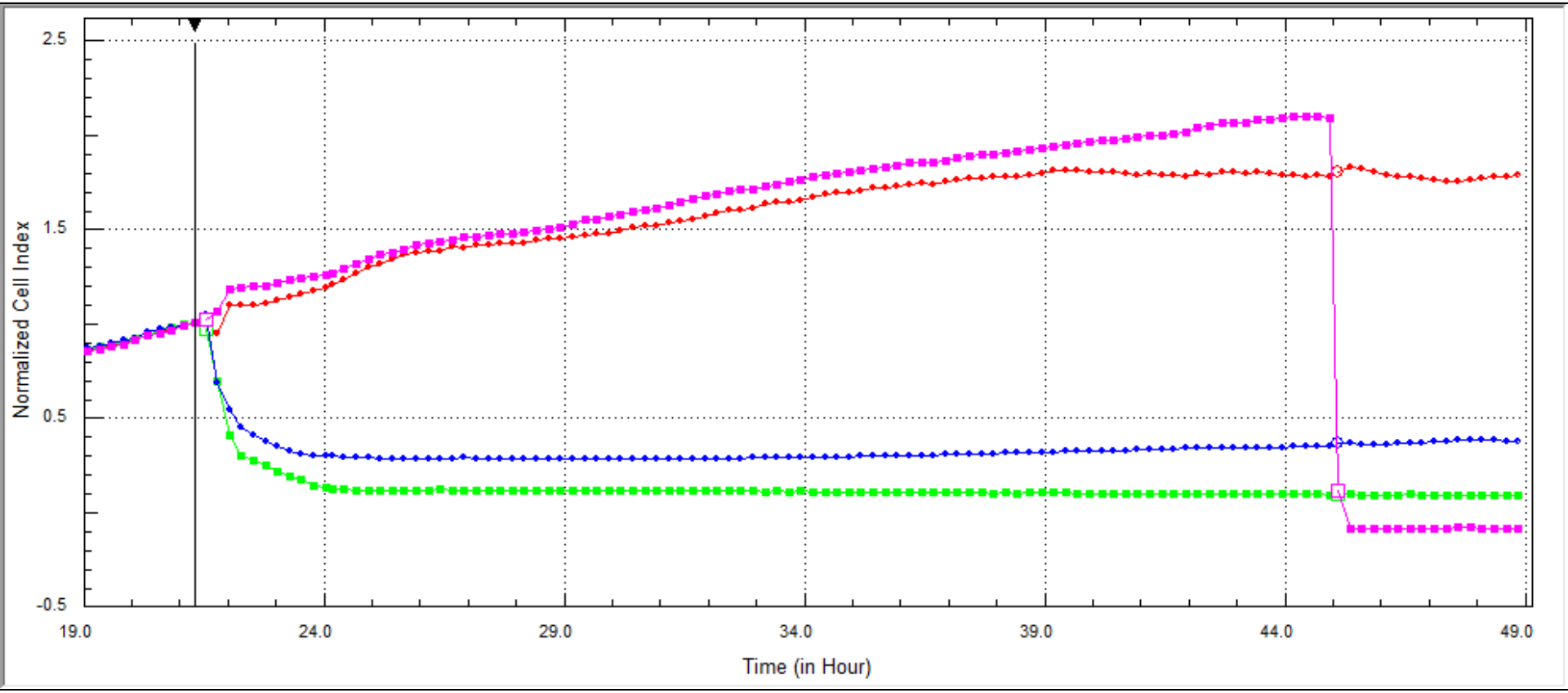
xCELLigence

- xCELLigence allows monitoring of adherent target cells by measuring electrical impedance of a current passed through the well
- Label-free System
- Cytotoxicity Time Course, rather than end-point Assay



1. Roche.com
John Dowling, unpublished

DNP xCELLigence Data



Control B16F10 (Unmodified)

Triton X Addition (Positive Control)

1 uM Staurosporine Treated B16F10s

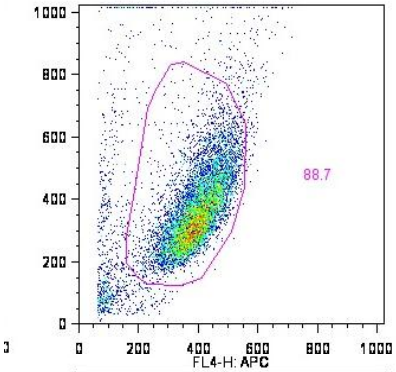
0.52 mM DNFB Treated B16F10s

Dan Erkes, unpublished

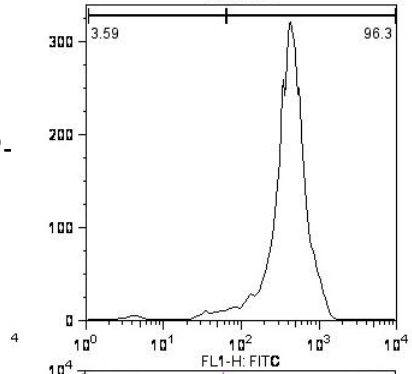
DNP-Mediated Cell Death: Annexin V/7-AAD

5.2 mM DNFB
B16F10

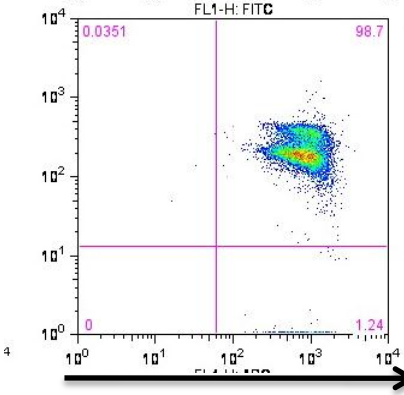
FSC x SSC



DNP+ vs. DNP-
(Total Cells)



Annexin V x
7-AAD (DNP+)

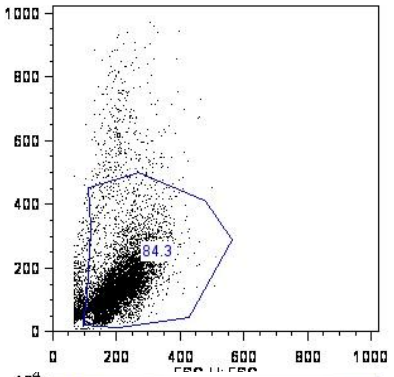


Annexin V

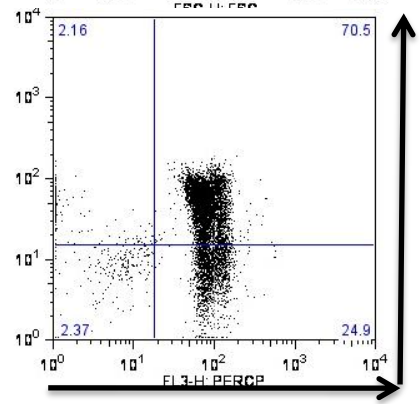
7-AAD

Saponin
(Permeabilization)
Control

FSC x SSC



Annexin V x
7-AAD (FITC+)



7-AAD

Annexin V

Dan Erkes, unpublished

DNP-Mediated Cell Death : Cleaved-Caspase 3

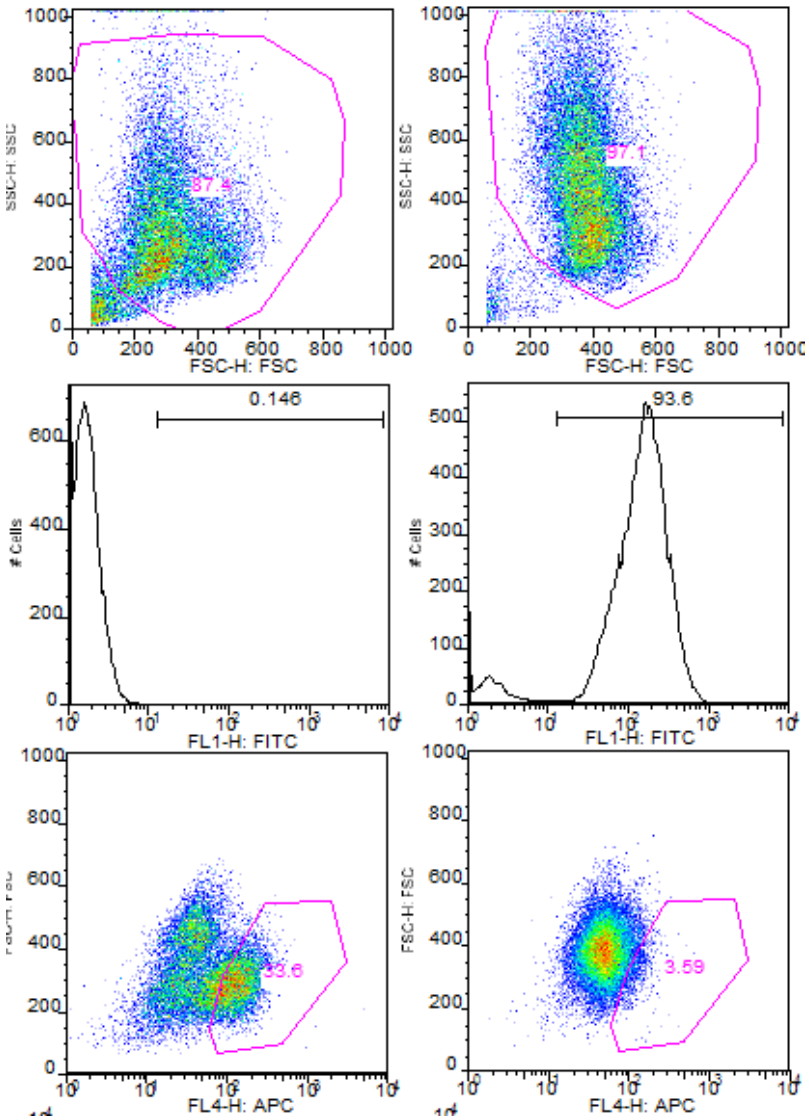
Staurosporine 1uM
24 hr Cleaved Caspase 3
(+) Control

0.52 mM DNP
Modified
B16F10s (3hrs)

FSC x SSC

DNP+ vs. DNP-
(Total Cells)

1° Cleaved- Caspase 3
2° Alexafluor 647

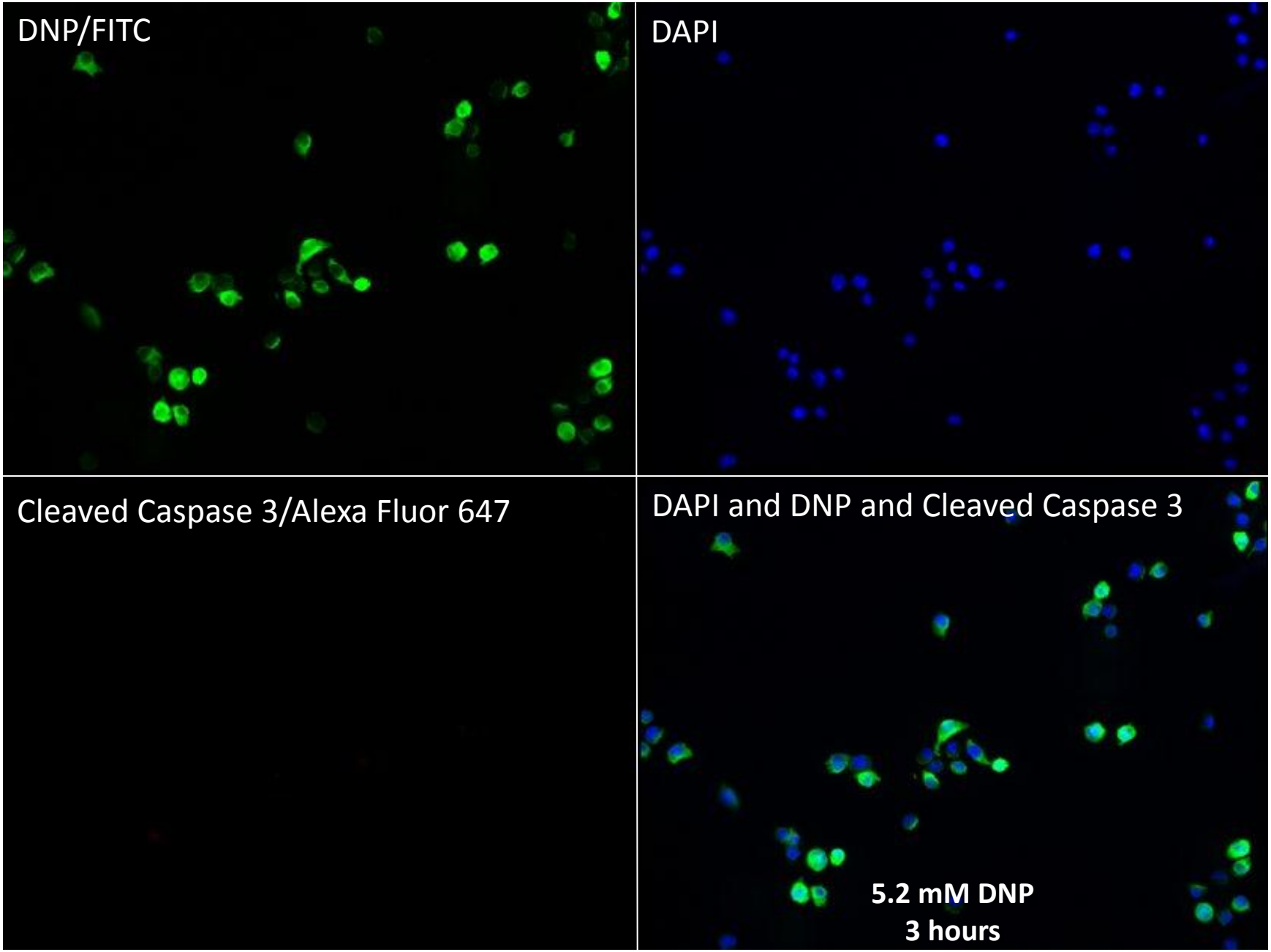


Dan Erkes, unpublished

1 uM Staurosporine added for 24 hours to B16F10 cells

Dan Erkes, unpublished

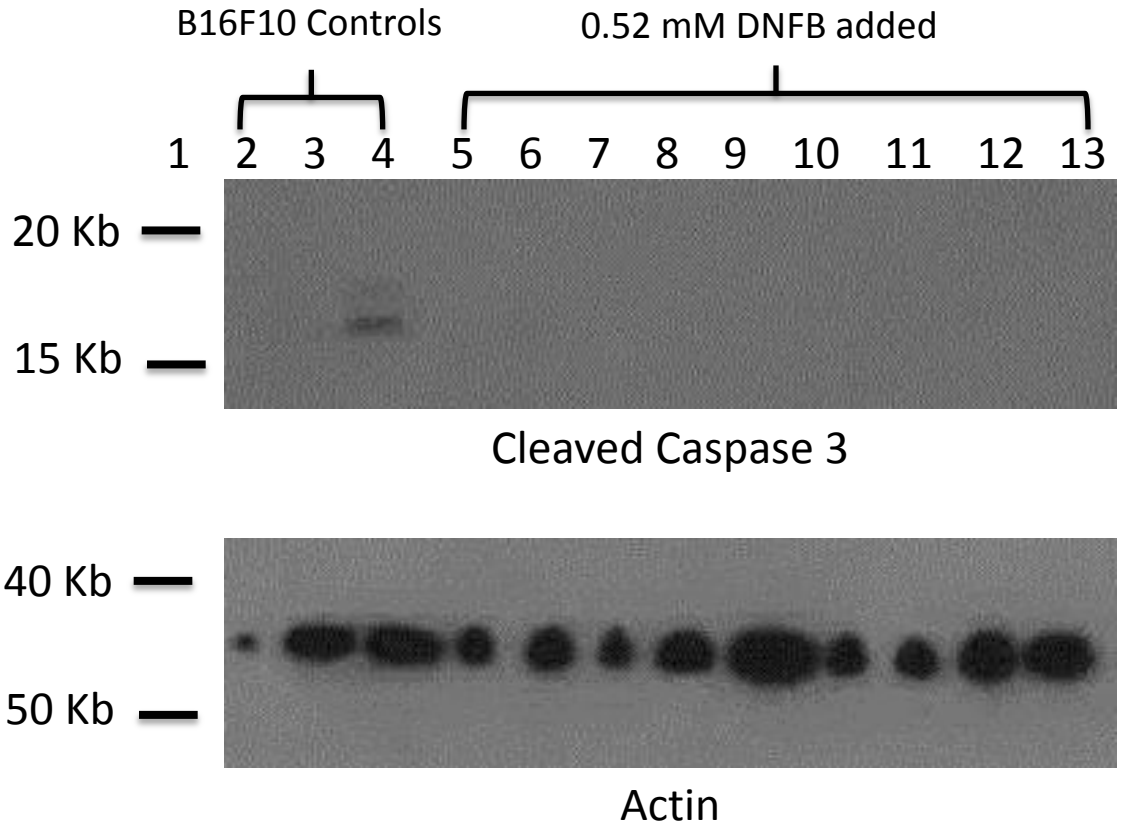
5.2 mM DNFB added to 4 million B16F10



Dan Erkes, unpublished

Cleaved- Caspase 3 and Cyclophilin A kinetics after DNFB exposure

1) Ladder
2) DNP (-) B16F10 Control
3) Ethanol 95% 30 Seconds
4) Staurosporine 1 uM 20 hrs
5) 0.52 mM DNFB 0.25 hours
6) 0.52 mM DNFB 0.5 hours
7) 0.52 mM DNFB 0.75 hours
8) 0.52mM DNFB 1 hours
9) 0.52 mM DNFB 1.5 hours
10) 0.52 mM DNFB 2 hours
11) 0.52 mM DNFB 3 hours
12) 0.52 mM DNFB 4 hours
13) 0.52 mM DNFB 6 hours



Dan Erkes, unpublished

Project Goals

- Modify B16F10 cells using 0.52 mM DNFB
- Incubate DNP-modified cells for various time points
- Collect these modified lysates and perform Western blotting
- Stain membranes with apoptotic and necrotic specific markers
- Infer what type of cell death is occurring in these cells and if this is time dependent

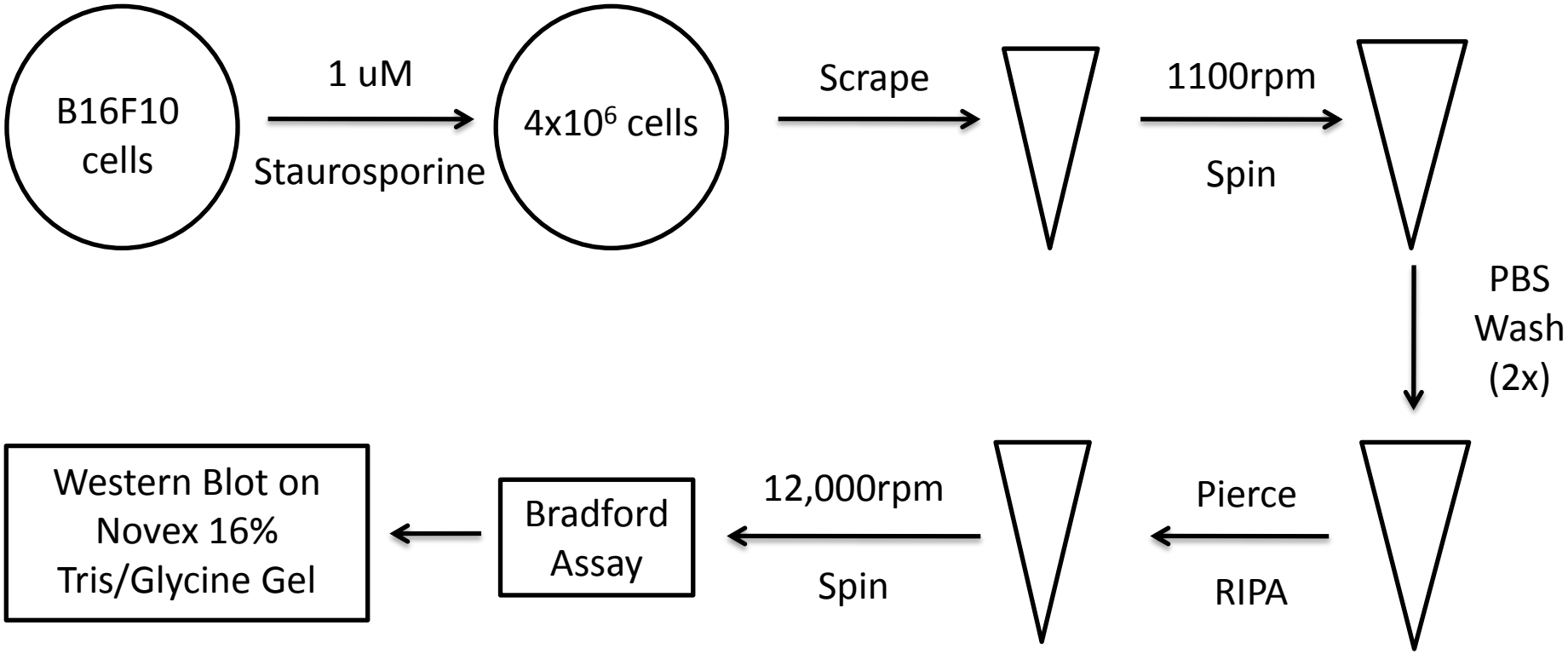
Apoptosis Control for My Experiments

Staurosporine Time Course

- Establish a positive apoptosis control for Cleaved Caspase-3
  - Staurosporine is a apoptosis inducer
- 1uM Staurosporine added to growing B16F10 cells
- Incubated at 37°C for the following time points: 4hr, 8hr, 12hr, 16hr, 20hr, and 24hr
- Western Blot performed on samples
  - Cell Signaling Cytochrome c treated Jurkat cells (apoptosis positive control)
  - Untreated Jurkat cells (apoptosis negative control)

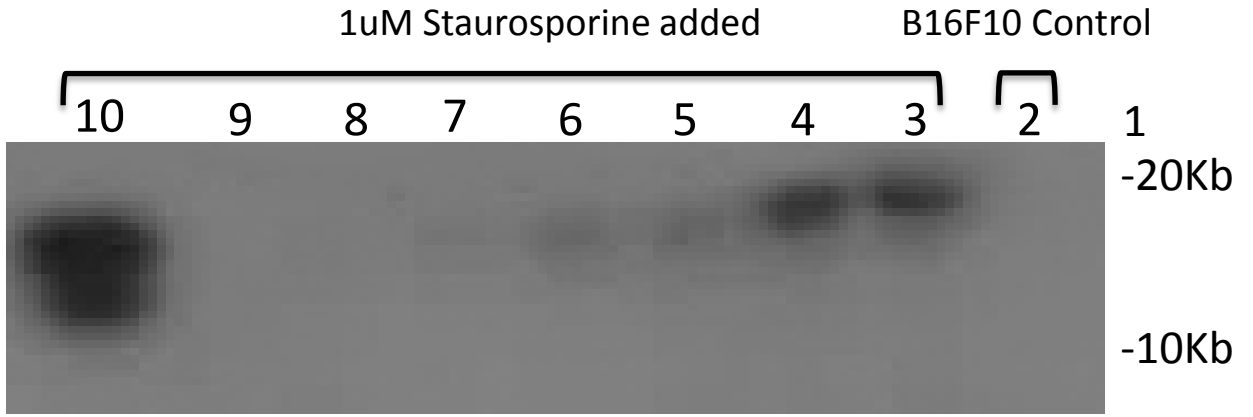
1. Enzo Life Sciences. Staurosporine Product Data Sheet

Staurosporine Lysate Collection

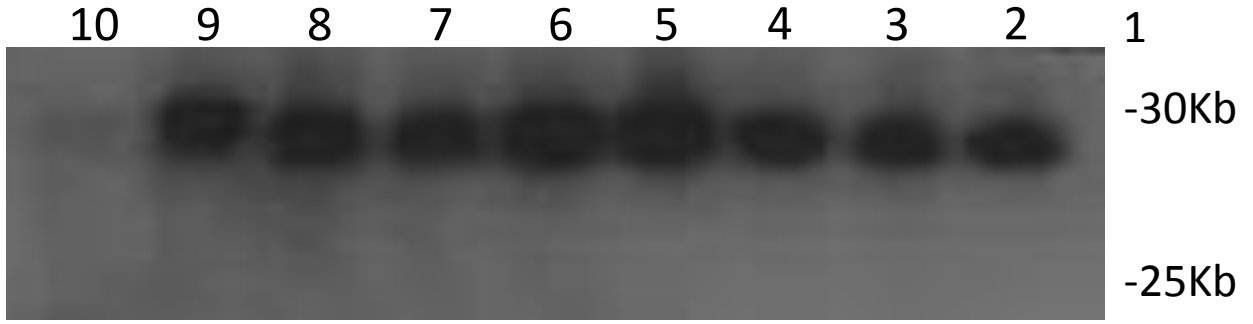


Cleaved Caspase-3 in Staurosporine Treated B16F10s

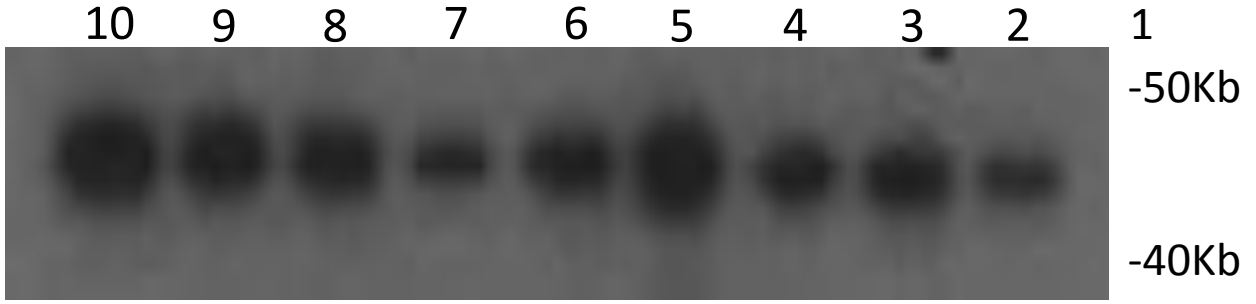
1) Ladder
2) Untreated B16F10 cells (negative control)
3) B16F10 cells treated with Staurosporine for 4hr
4) B16F10 cells treated with Staurosporine for 8hr
5) B16F10 cells treated with Staurosporine for 12hr
6) B16F10 cells treated with Staurosporine for 16hr
7) B16F10 cells treated with Staurosporine for 20hr
8) B16F10 cells treated with Staurosporine for 24hr
9) Untreated Jurkat Cells (negative apoptosis company control)
10) Cytochrome C Jurkat Cells (positive apoptosis company control)



Cleaved Caspase-3 (5.5hr Exposure)



Pro-Caspase-3 (5.5hr exposure)



Anti-Actin (10min exposure)

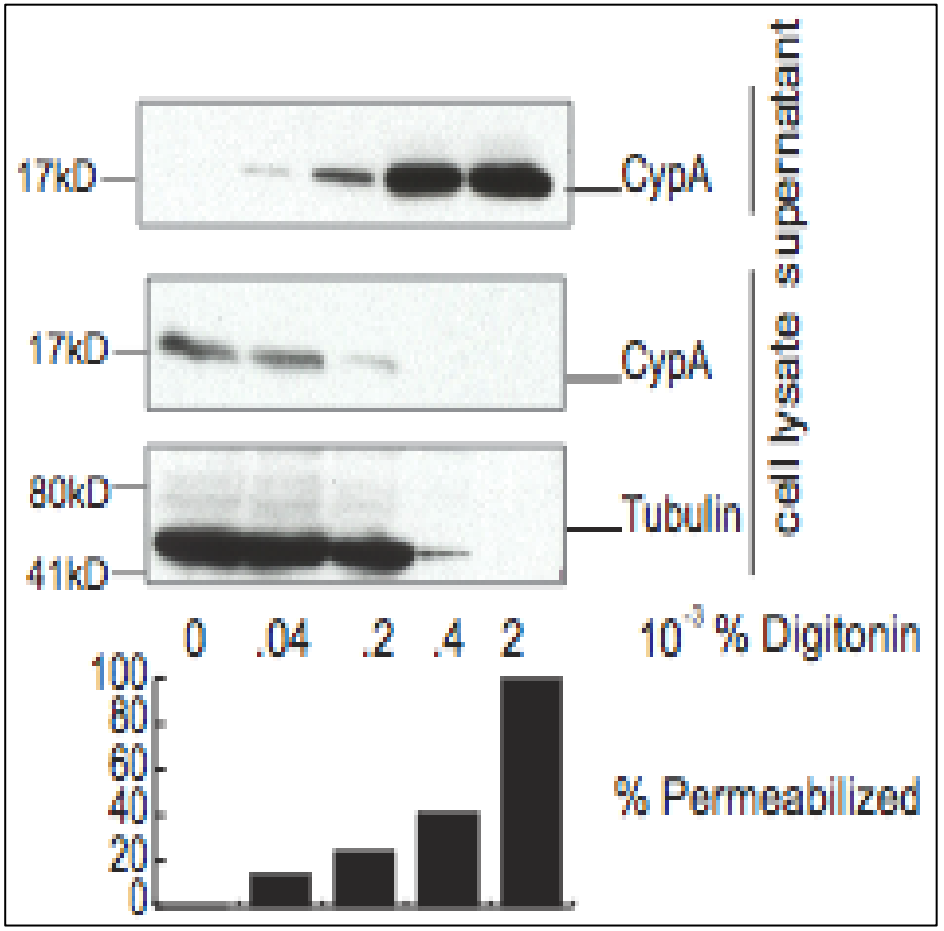
Conclusions

- Staurosporine is an effective positive control for apoptosis
- 4 hr exposure gives the strongest Cleaved Caspase-3 signal in B16F10 cells
- 4 hr Staurosporine sample will be used as an apoptosis positive control for future Western blot experiments

Necrosis Control for my Experiments

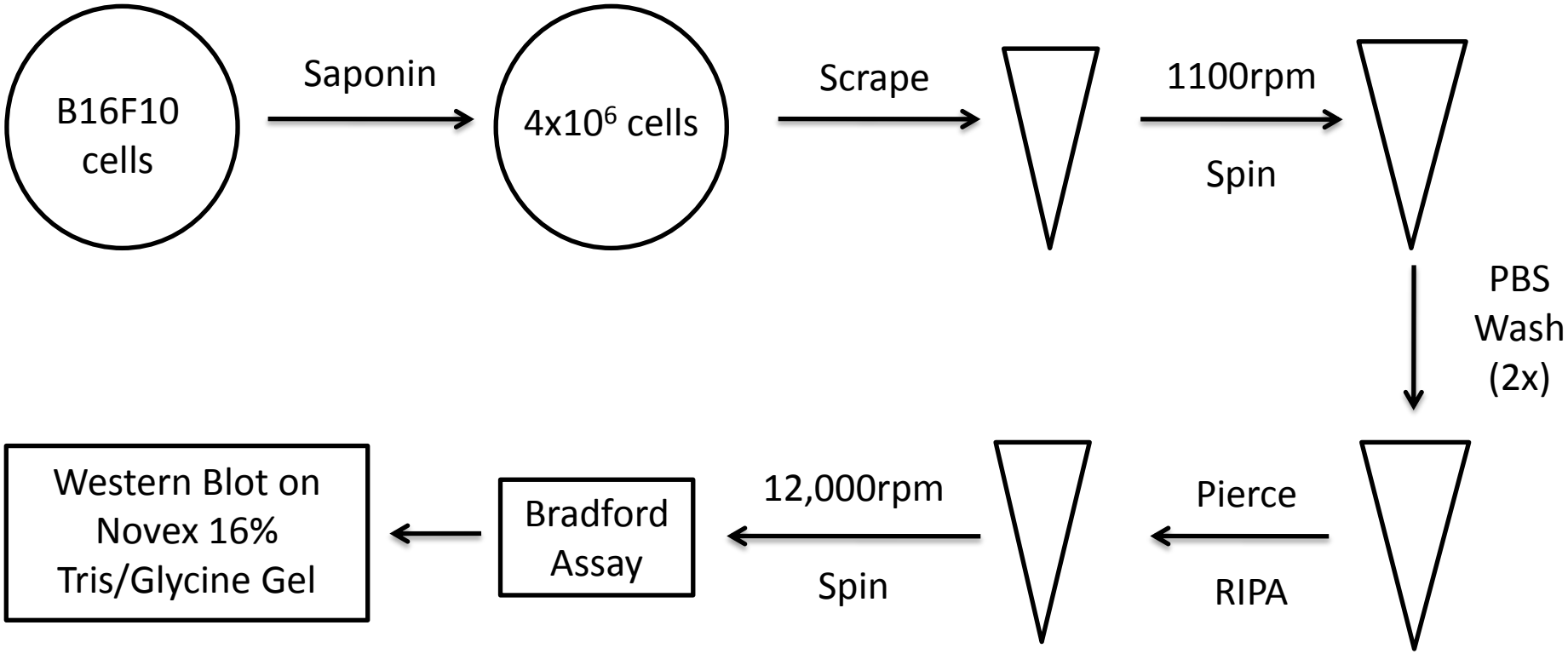
Saponin Serial Dilutions

- Establish positive necrosis control
- Detergent
- Protocol based off of Christofferson and Yuan, 2010
  - Thought that DNFB functions similarly; permeabilization
- All samples incubated at room temp. for 5min with respective Saponin dilutions: 0.1%, 0.02%, 0.004%, 0.0008%, and 0.00016%
- Check cell permeabilization and Cyclophilin A expression

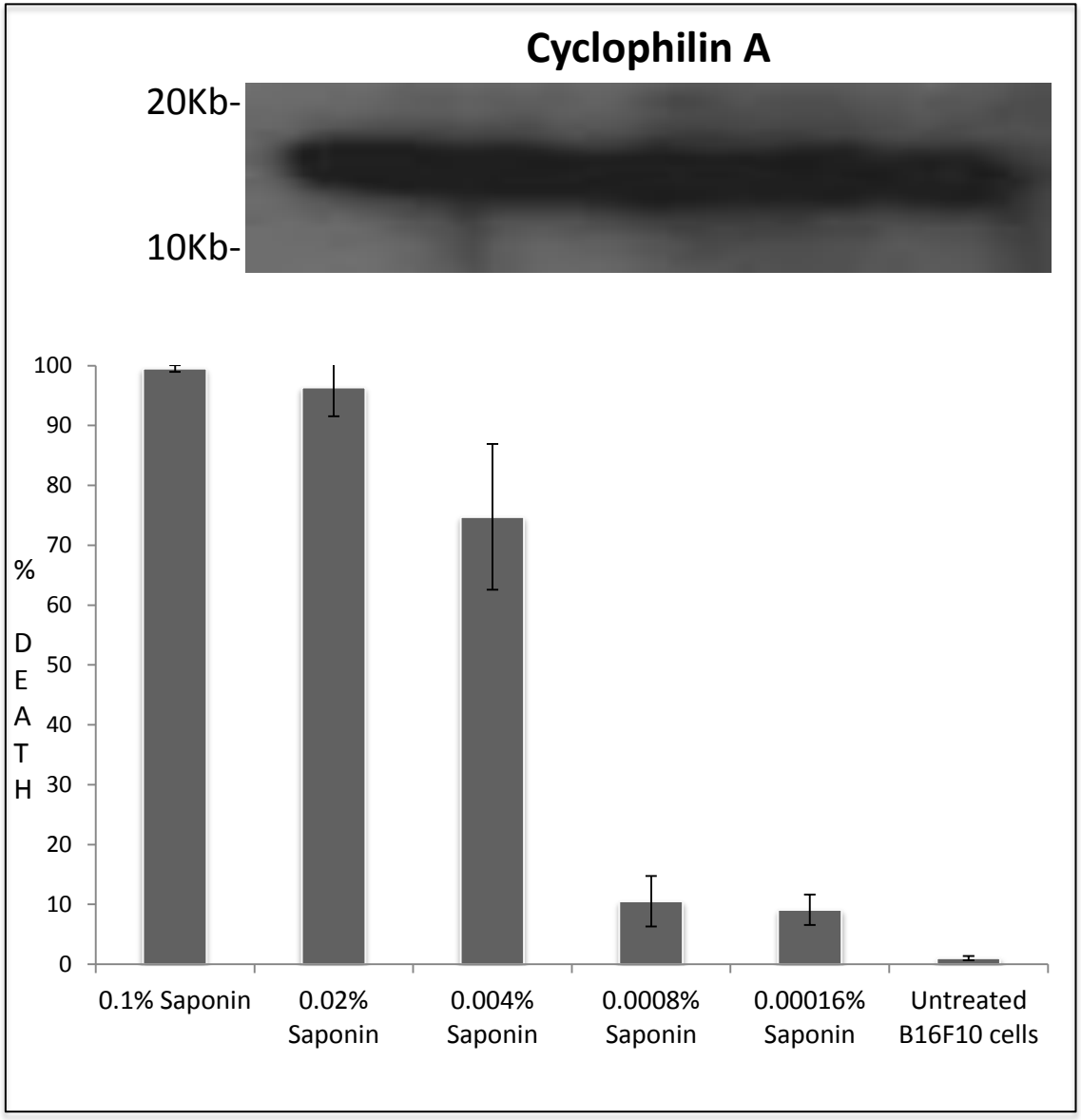


Christofferson and Yuan. 2010. *Cell Death Differ*. 17 (12): 1942-1943

Saponin Lysate Collection



Saponin Results



Percent Death
=
Percent Permeabilized

Conclusions

- Saponin is an ineffective positive control for necrosis
  - Cyclophilin A is present at all concentrations
- Did not have enough time to create a good necrosis positive control
- Experiments had to be run without a necrosis positive control
- Examine the possible necrotic controls
  - Digitonin
  - Triton X

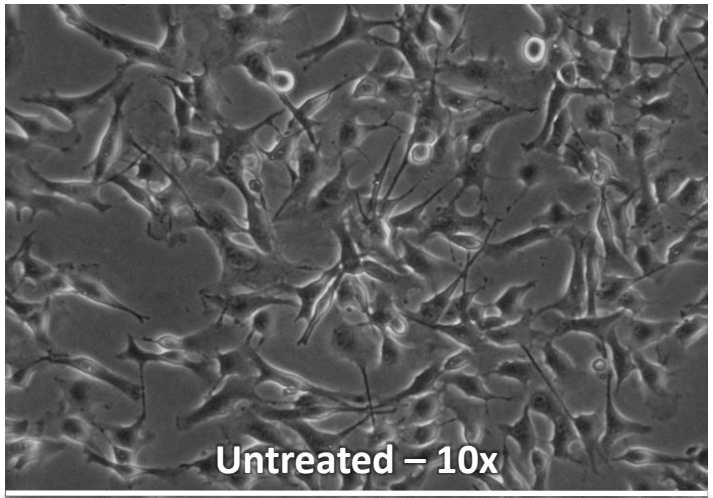
Project Goals

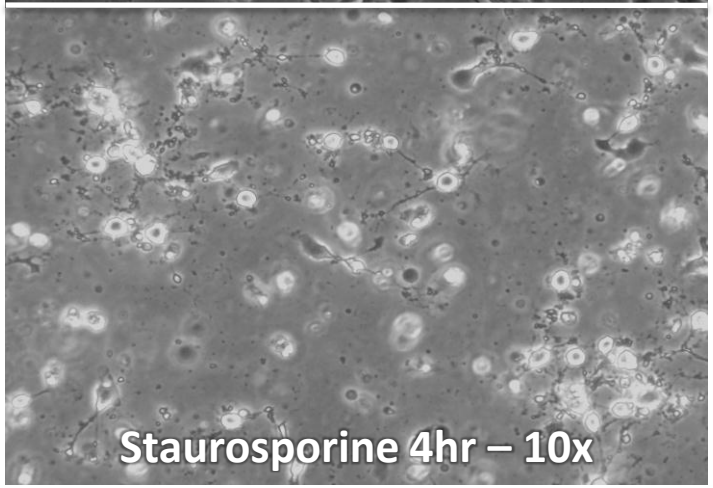
- **Modify B16F10 cells using 0.52 mM DNFB**
- **Incubate DNP-modified cells for various time points**
- Collect these modified lysates and perform Western blotting
- Stain membranes with apoptotic and necrotic specific markers
- Infer what type of cell death is occurring in these cells and if this is affected by time

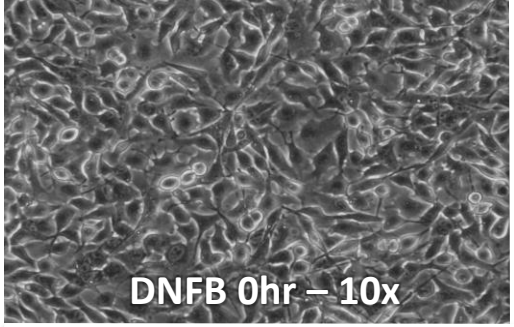
DNP-mediated Cell Death Time Course

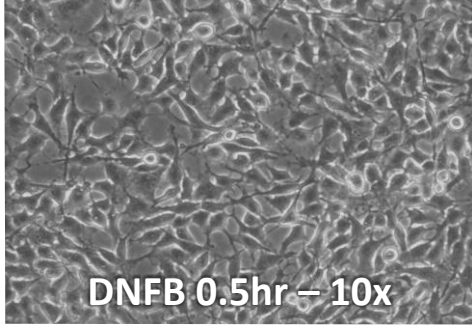
- Determine the mechanism of DNP-mediated cell death in B16F10 cells
- 0.52 mM of DNFB added to healthily growing B16F10 cells
- Incubated at 37°C for the following times: 0hr, 0.25hr, 0.5hr, 1hr, 1.5hr, 2hr, 3hr, 4hr, 6hr, 8hr, 12hr, and 24hr.
- Western Blot performed on samples against B16F10 cells and Staurosporine apoptosis control

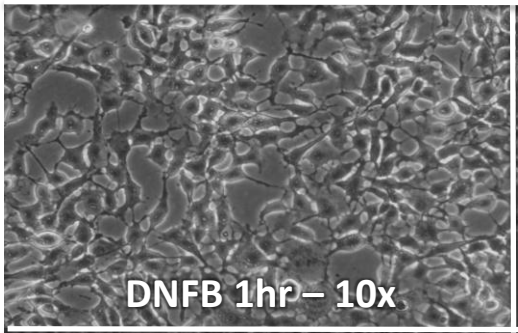
Morphology of DNP-Modified B16F10 Cells

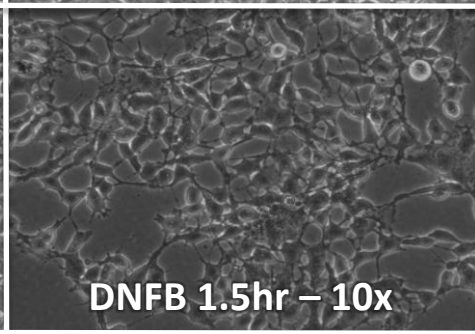


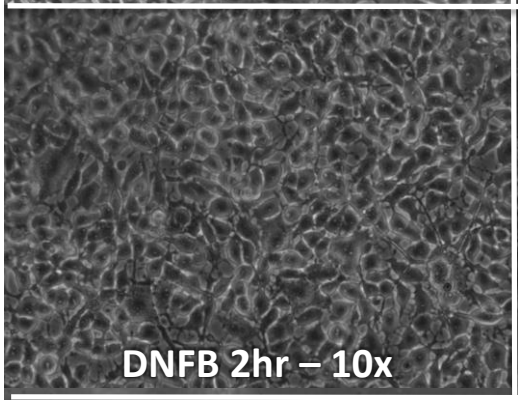


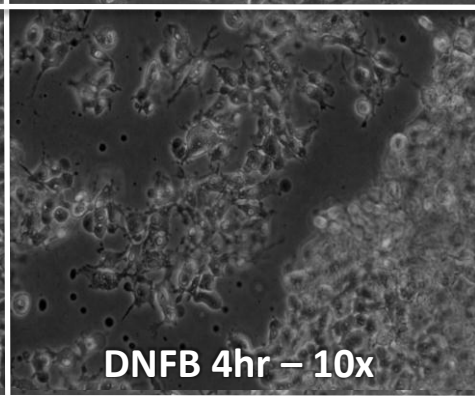


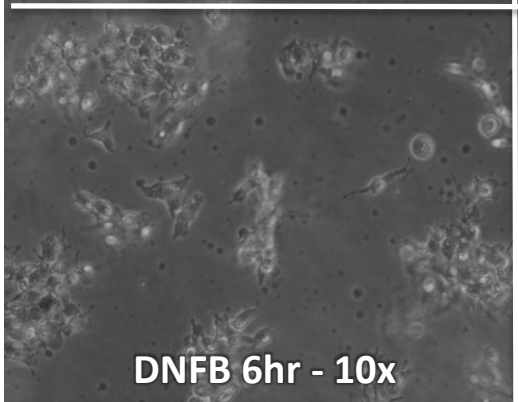


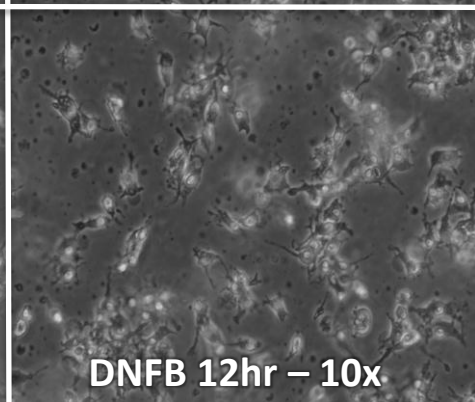








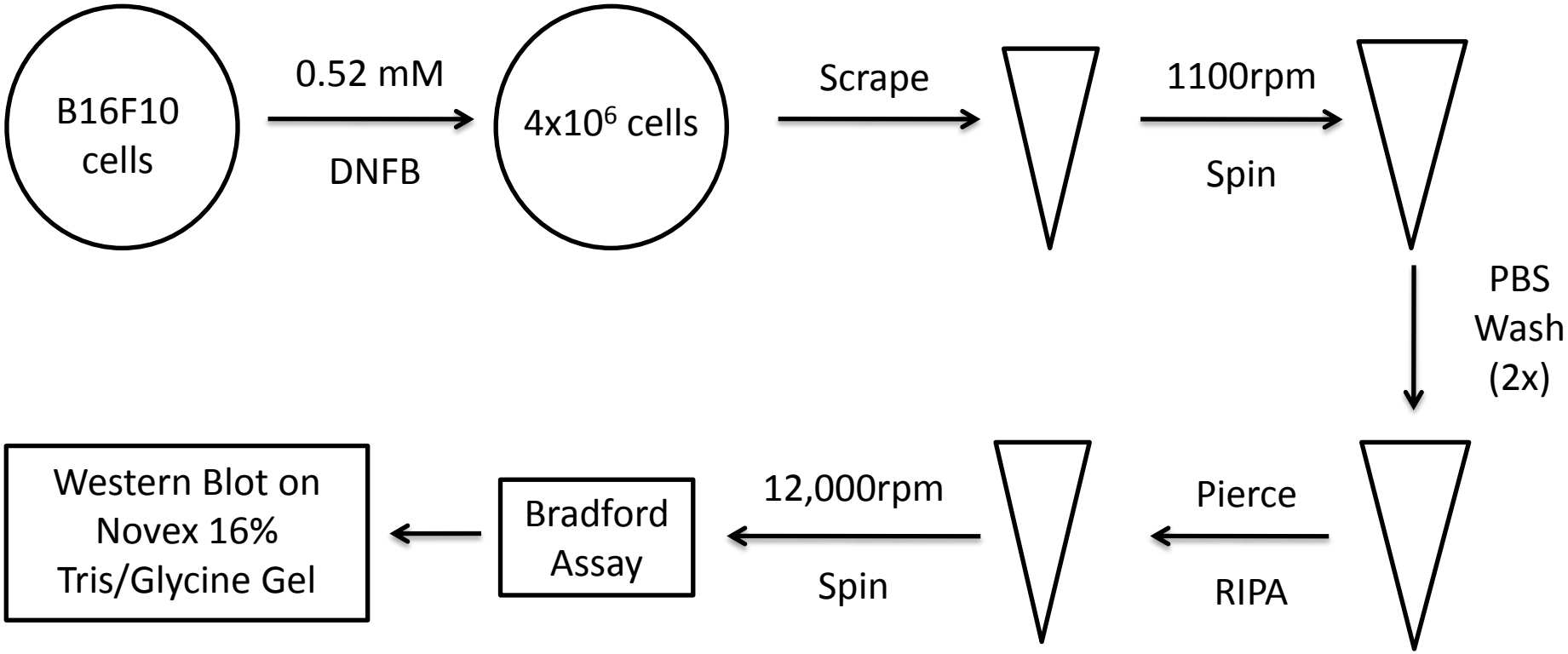




Project Goals

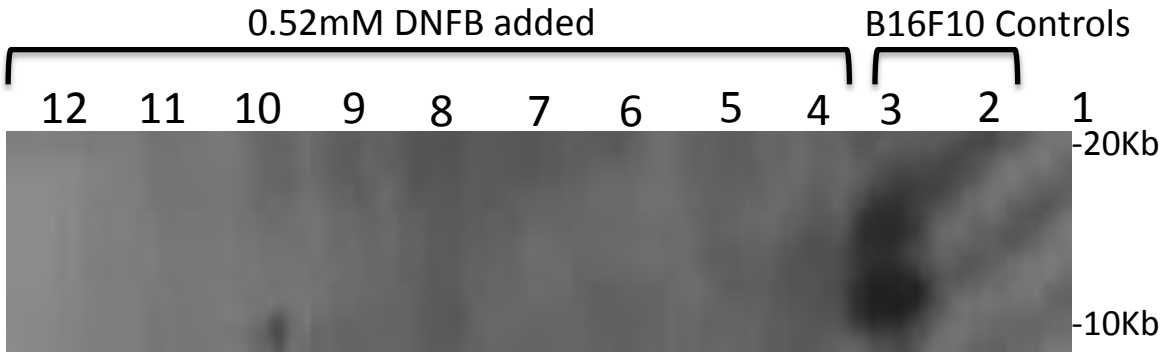
- Modify B16F10 cells using our 0.52 mM DNFB
- Incubate DNP-modified cells for various time points
- **Collected these modified lysates and perform Western blotting**
- **Stain membranes with apoptosis and necrosis specific markers**
- **Infer what type of cell death is occurring in these cells and if this is affected by time**

DNFB Lysate Collection

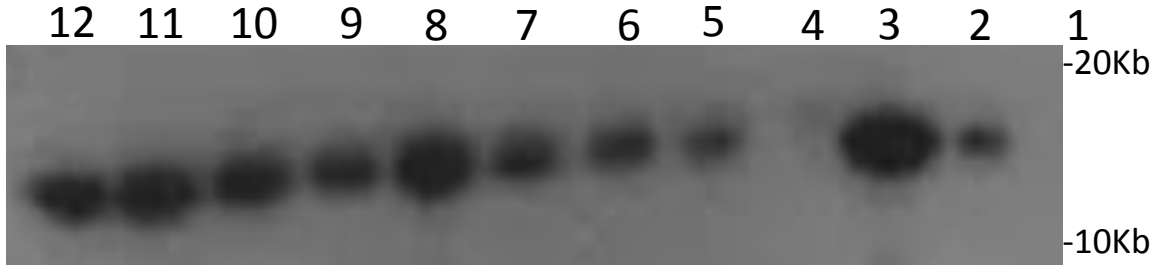


Time Kinetics of DNFB Lysates for Cleaved Caspase-3 and Cyclophilin A (DE Lysates)

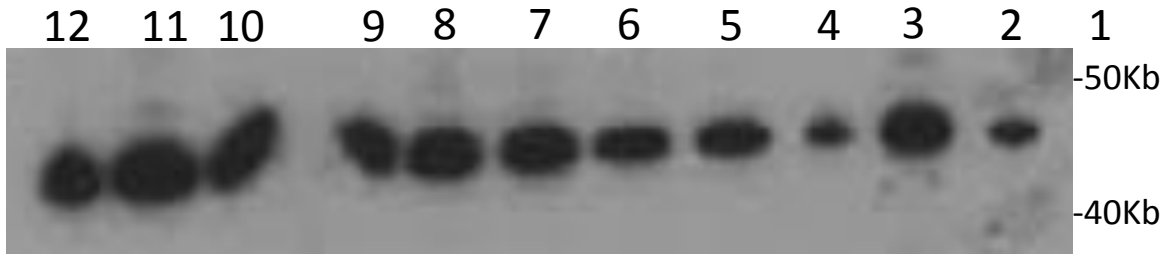
1) Ladder
2) DNP (-) B16F10 Control
3) Staurosporine 1 uM 20 hrs
4) 0.52 mM DNFB 0.25 hours
5) 0.52 mM DNFB 0.5 hours
6) 0.52 mM DNFB 0.75 hours
7) 0.52 mM DNFB 1 hours
8) 0.52 mM DNFB 1.5 hours
9) 0.52 mM DNFB 2 hours
10) 0.52 mM DNFB 3 hours
11) 0.52 mM DNFB 4 hours
12) 0.52 mM DNFB 6 hours



Cleaved Caspase-3 (5.5hr)



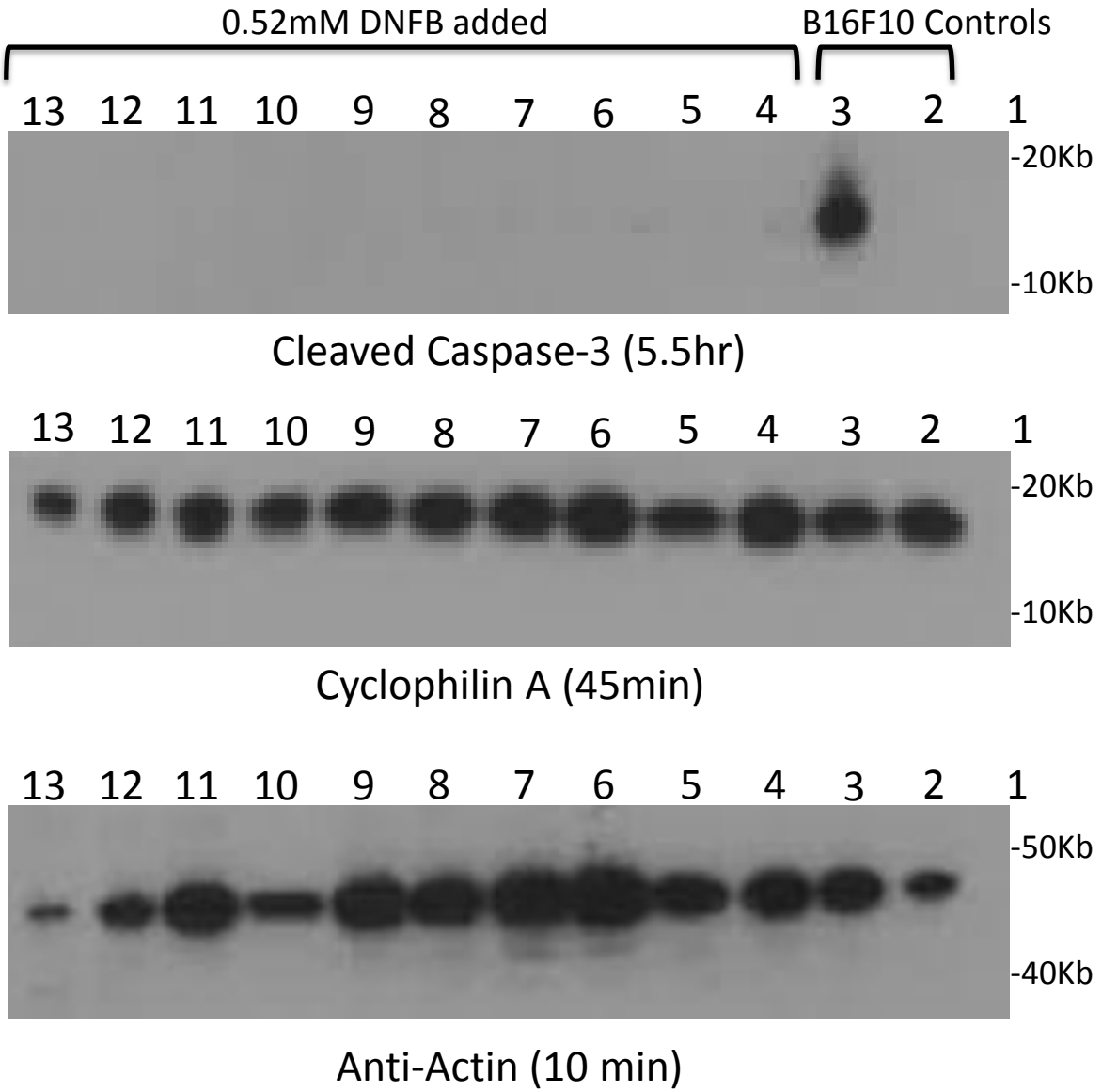
Cyclophilin A (20min)



Anti-Actin (10min)

Time Kinetics of DNFB Lysates for Cleaved Caspase-3 and Cyclophilin A (FT Lysates)

1)Ladder
2)DNP (-) B16F10 Control
3)Staurosporine 1 uM 4 hrs
4) 0.52 mM DNFB 0 Hours
5) 0.52 mM DNFB 0.25 Hours
6) 0.52 mM DNFB 0.5 Hours
7) 0.52 mM DNFB 0.75 Hours
8) 0.52 mM DNFB 1 Hour
9) 0.52 mM DNFB 1.5 Hours
10) 0.52 mM DNFB 2 Hours
11) 0.52 mM DNFB 3 Hours
12) 0.52 mM DNFB 4 Hours
13) 0.52 mM DNFB 6 Hours



Discussion

- Absence of Cleaved Caspase-3 in both untreated cells and DNFB-treated cells indicates that the caspase-dependent apoptotic pathway is not being activated
- Cyclophilin A appears to be expressed in untreated B16F10s, Staurosporine treated B16F10s, and when treated with DNFB
  - Test for the presence of HMGB1
  - Further investigation into necrosis is needed as a positive control was unattained – Possibly Digitonin or Triton X
  - Experiment will need to be repeated
- If necrosis is not occurring, DNFB could cause the cells to enter:
  - Pyrotopsis – IL-1B, HMGB1, and Caspase-1 release
  - Pyronecrosis – IL-1B, HMGB1 release
- Mechanism of DNP-mediated cell death still remains unclear

Kroemer, G., et al. *Cell Death Differ*. 2009. 16 (1): 3-11.

Acknowledgements

- **Dan Erkes, PhD. Scholar – Immunology**
- Dr. Makoto Yoshida, PhD.
- Dr. Mizue Terai, PhD.
- John Dowling, PhD. Scholar – Immunology
- Dr. Shinji Ozaki, MD., PhD.
- Dr. Ken Kageyama, MD., PhD.

- Dr. Takami Sato, MD., PhD. and Dr. Senthamil Selvan, PhD.

- Dr. Scott Waldman, MD., PhD.
- Joy Soleiman, MPA
- Dr. Catalina Arango, PhD.

SAINT JOSEPH'S

Jefferson™
THOMAS JEFFERSON UNIVERSITY