



ThermoFisher
S C I E N T I F I C

NIST-FDA Cell Counting Workshop: Sharing practices in cell counting measurements

April 10, 2017

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ThermoFisher Scientific

Cell Counting| Counter Considerations

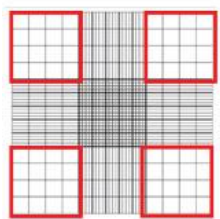


Figure 1: Hemacytometer grid

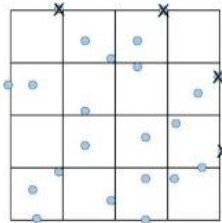
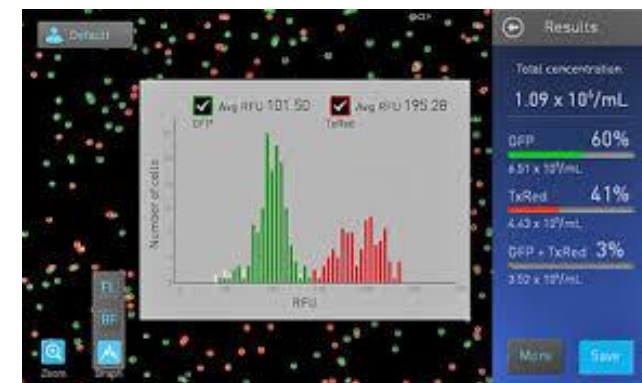


Figure 2: Cell counting guideline



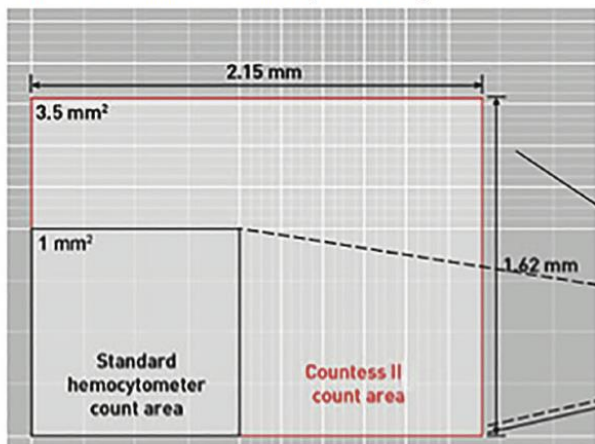
- Manual
- Variable
- Personal bias affects count

- Automated
- 10^4 - 10^7 Concentration and 3-25 μ M size range accuracy
- Brightfield count only

- Automated
- 10^4 - 10^7 Concentration and 3-25 μ M size range accuracy
- Brightfield and Fluorescence capabilities

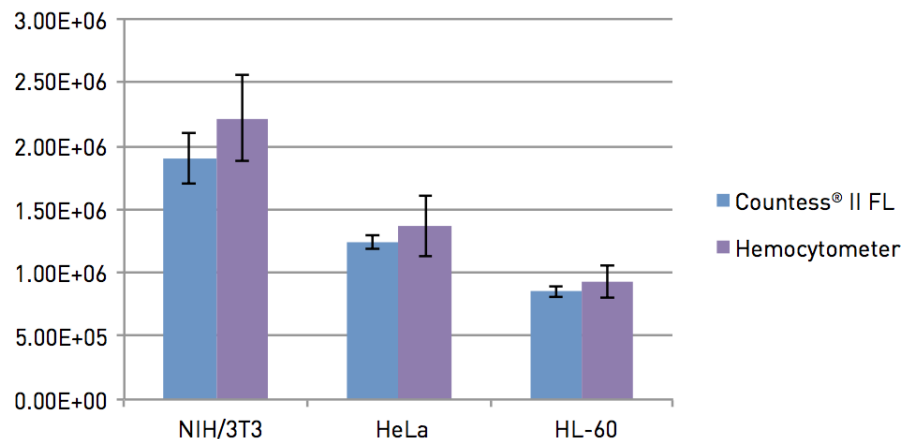
Cell Counting | Measurement Considerations

Area: 2.15 mm x 1.62 mm (3.48 mm²)

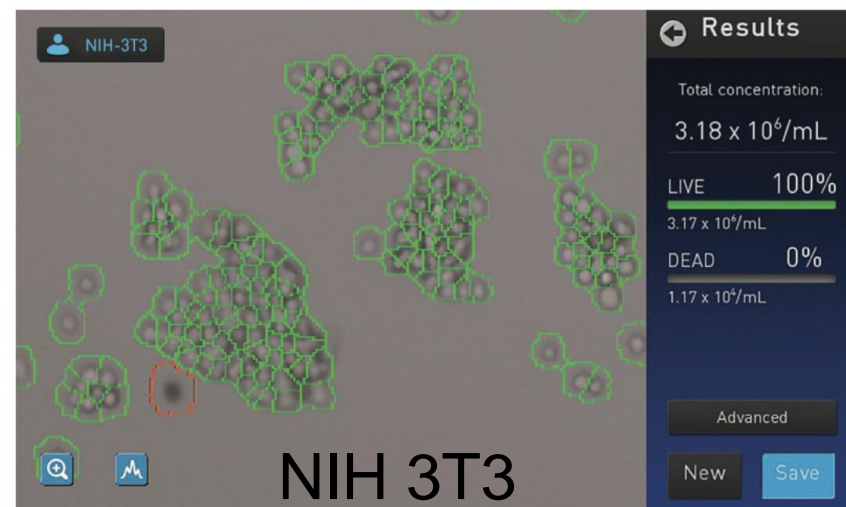
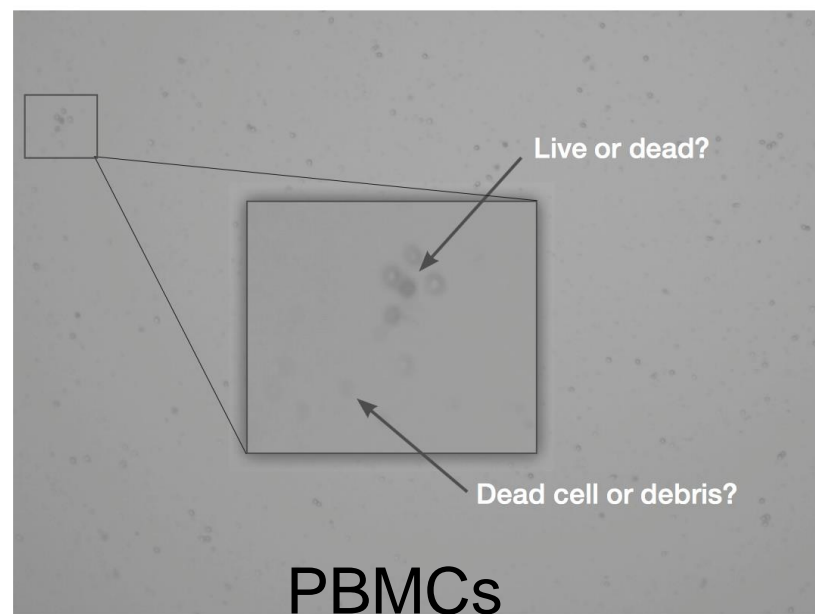
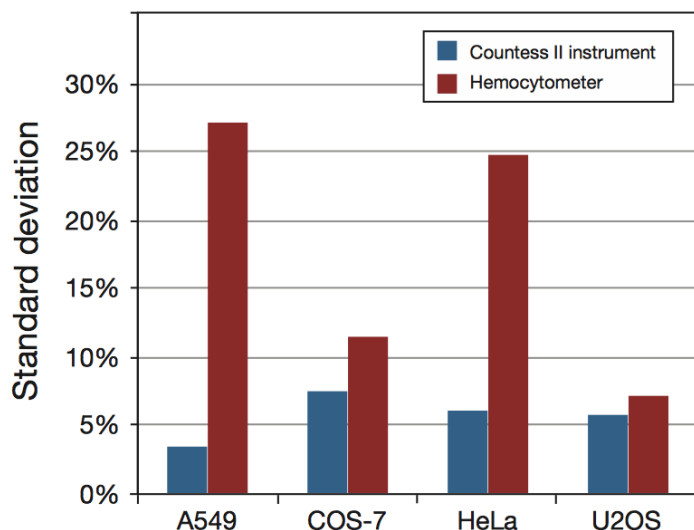


- Reusable Hemacytometer
- Dedicated machine slides

- Test and retest to users gold standard
- Automated faster over larger area
- Options matter for users- “Trust but verify” cell count

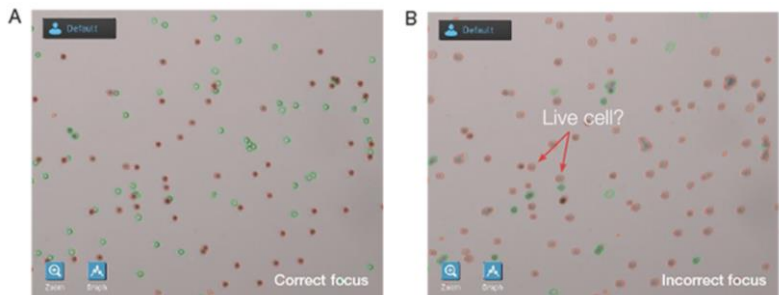


Cell Counting | Cell prep and variability



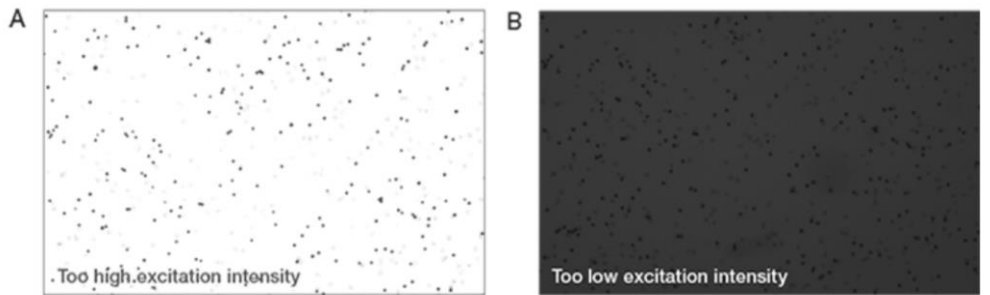
- Higher variability with manual counting
- Sample prep continues to be an issue
- Size variability within sample
- Autofocus and counting algorithms improve accuracy and repeatability

Cell Counting | User Needs and Variability

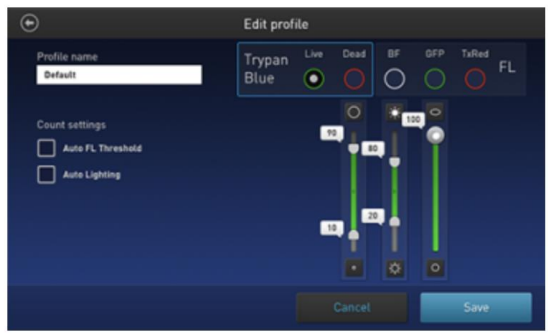


Focusing - Get what you “auto focus” for

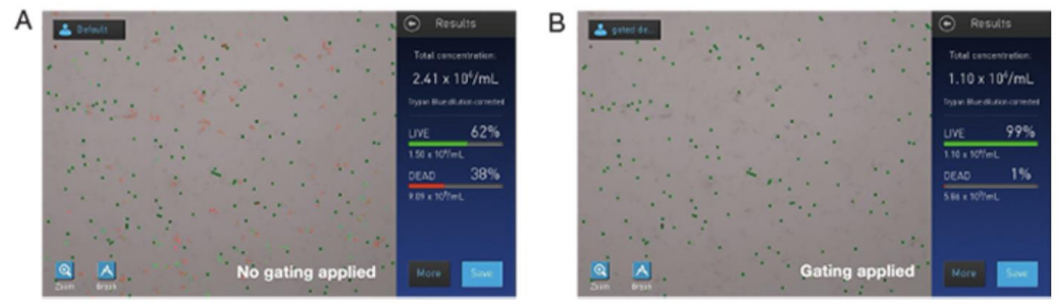
- Users need flexibility-But introduces variability
- Gating on size, circularity, brightness, and fluorescent intensity
- Training and examples
 - Applications and White Papers
 - Field Support
- Ability to customize and save protocols



Light intensity- Difficult to predict what users will do

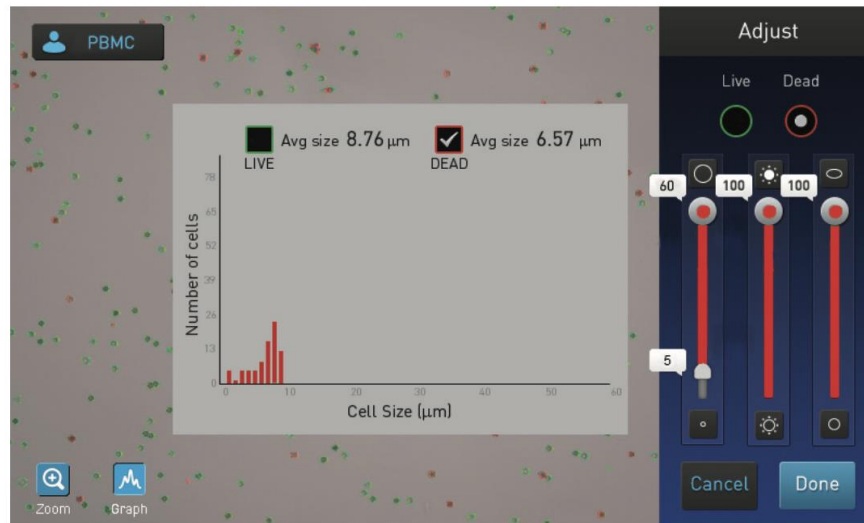
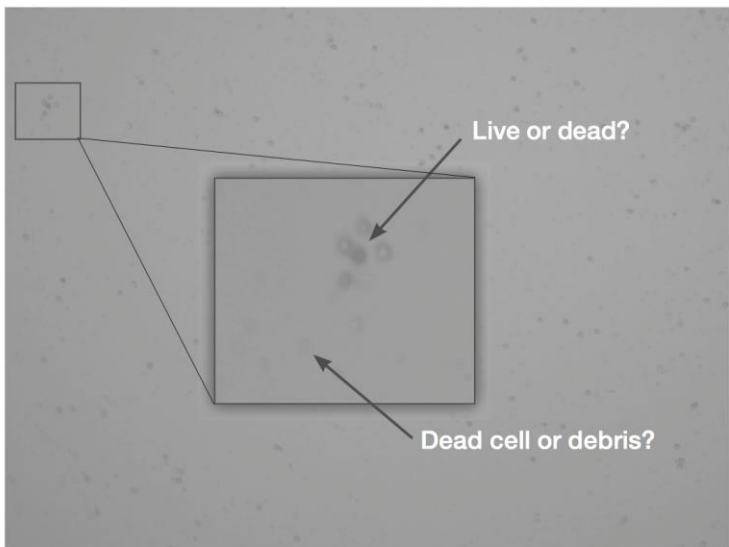


Gating in or out of cell populations

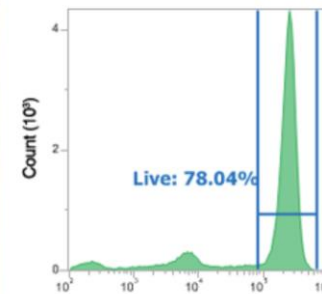
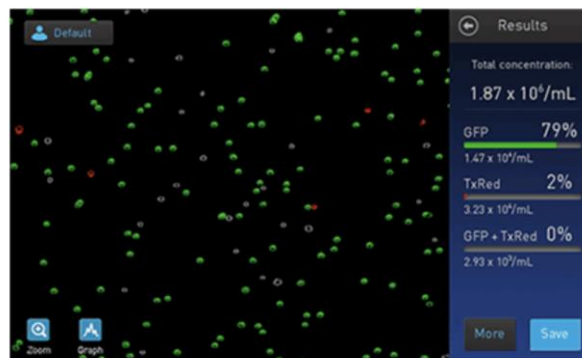


Flexibility allows for options and better cell counts

Cell Counting | PBMC User Issues



- Difficult for users because of sample variability and differences between preps
- Precious sample with limited quantity
- Provide “Flow-Like” gating capability for inclusion/exclusion
- Cross validate between instruments



Automated Counter Flow Cytometer

Cell Counting | Stem Cell User- iPSC to Cardiomyocyte

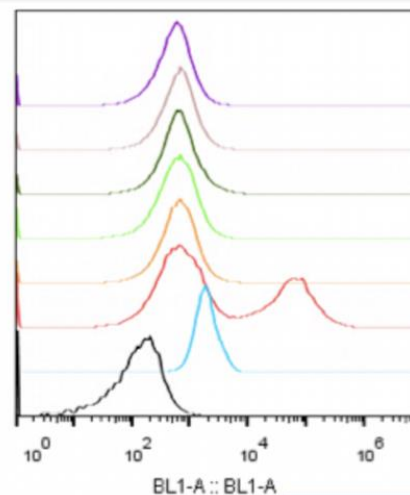
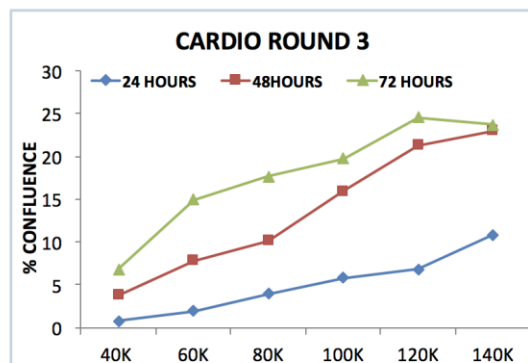
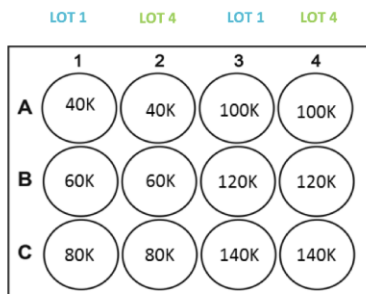
Operator	Hemocytometer			Viability	Countess 1				Countess 2				
	Total	Live	Dead		Total	live	Dead (10E6)	Viability	total	live	dead	viability	
Chad	1.61	1.57		0.04	98	1.3*10E6	1.2*10E6	0.12	91%	1.09	0.962	0.129	88%
	1.575	1.5		0.075	95	1.4*10E6	1.4*10E6	0.07	95%	1.51	1.28	0.229	85%
Rene	1.73	1.655		0.075	96	1.7*10E6	1.5*10E6	0.13	92%	1.48	1.16	0.328	78%
	1.925	1.88		0.045	98	1.6*10E6	1.5*10E6	0.13	92%	1.61	1.26	0.352	78%
Maha	2.17	2.03		0.14	94	1.3*10E6	1.2*10E6	0.09	93%	1.22	1.04	0.182	85%
	2.05	1.96		0.09	96	0.87*10E6	0.82*10E6	0.05	94%	0.803	0.68	0.117	85%

CELL COUNTS

COUNTLESS 1 L: 2.55E+06; V: 89%

COUNTLESS 2 L: 3.1E+06; V: 91%

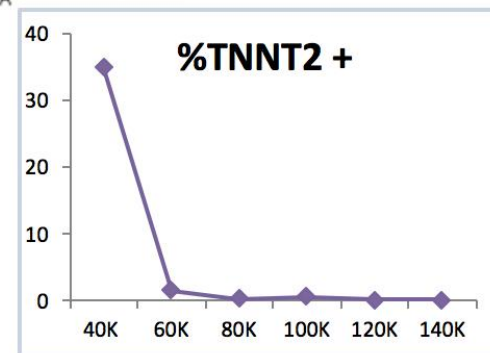
CONF	40K	60K	80K	100K	120K	140K
24 HOURS	0.7712	1.9145	3.9441	5.7771	6.8433	10.803
48 HOURS	3.7933	7.7911	10.127	15.979	21.279	22.995
72 HOURS	6.8057	14.914	17.634	19.747	24.53	23.668



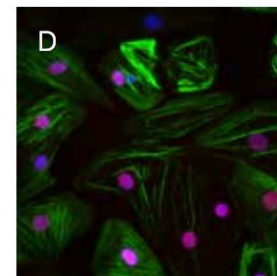
Sample Name	
█	LOT1 140K.fcs
█	LOT1 120K.fcs
█	LOT1 100K.fcs
█	LOT1 80K.fcs
█	LOT1 60K.fcs
█	LOT1 40K.fcs
█	iPSC TNNT2.fcs
█	LOT1 UNSTAINED.fcs

Flow Data

Cell Den.	TNNT2 +
40K	35
60K	1.61
80K	0.31
100K	0.61
120K	0.25
140K	0.17

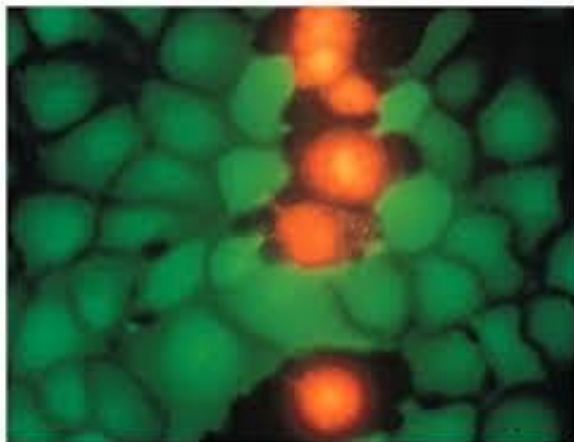


- Counting for original Seeding of iPSCs critical to Cardiomyocyte differentiation
- 40K in 12 well plate gives optimal results

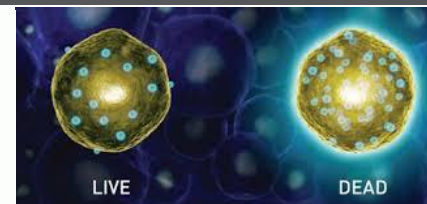
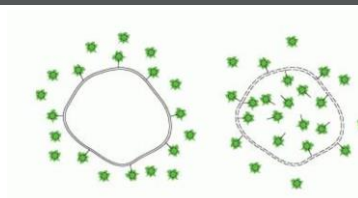


Imaging of TnT

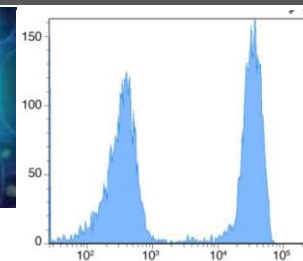
Cell Counting | Cell Health



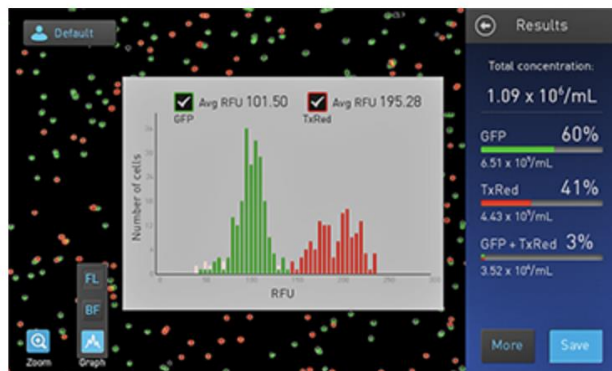
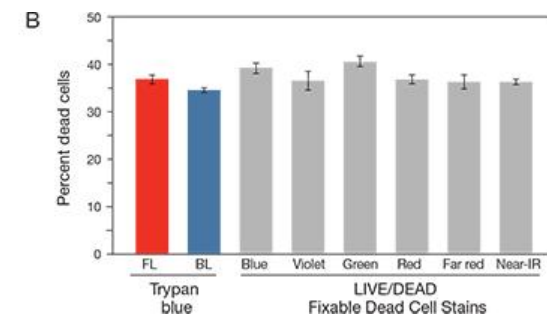
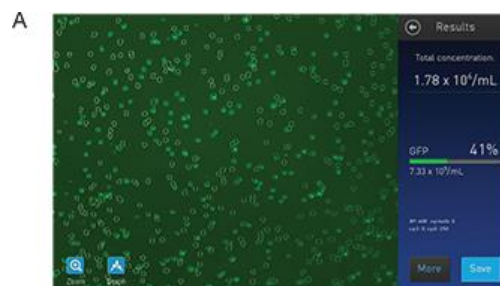
Live/Dead - Metabolism/Membrane Integrity



Fixable Live/Dead - Membrane Integrity



Flow Cytometry



- Cell health important for downstream uses
- Need accurate cell #s and accurate picture of cell health
- Traditional imaging and flow cell health reagents can be used

Continuum of Cell Health

Viable

- RNA/Protein quality control
- Polarized mitochondria
- [ATP] high
- Reducing cytoplasm
- Regulated proliferation
- Morphology

Apoptotic

- Caspase activation
- DNA Damage
- Removal of DAMPs

Necrotic/Necroptotic

- RIP kinase (PCD)
- DAMPs present

Pre-lethal

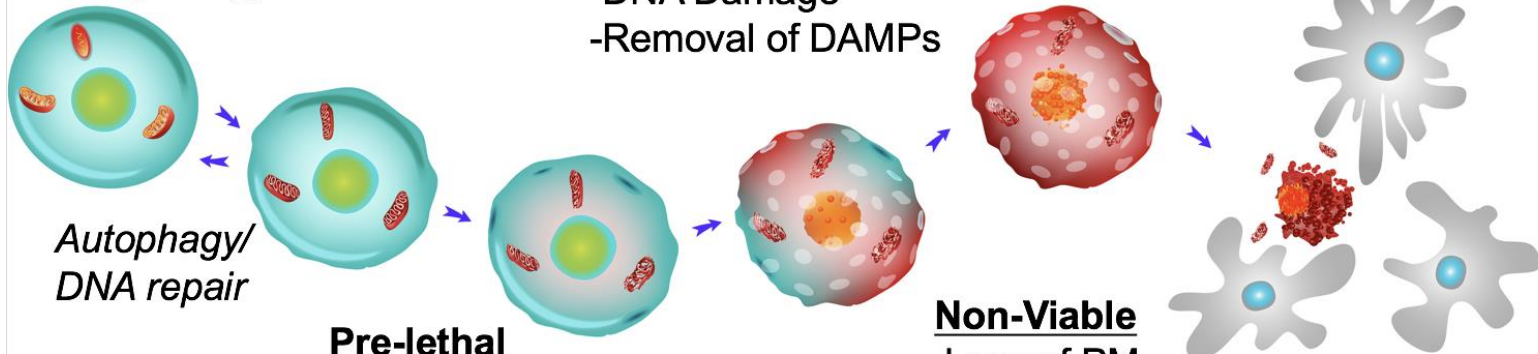
- Loss of protein quality control
- Depolarized mitochondria
- Oxidizing cytoplasm
- [ATP] low
- Oxidative/nitrative stress
- Perturbation of lipid metabolism
- Deregulated proliferation

Non-Viable

- Loss of PM integrity

Immunological consequence

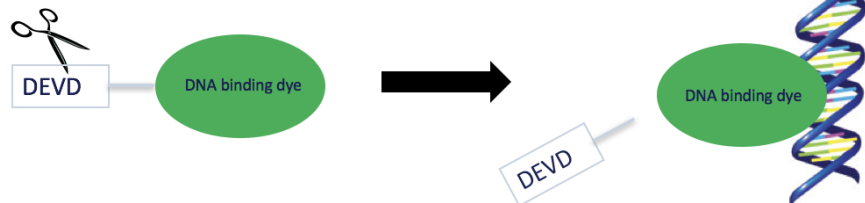
- Phagocytosis
- Inflammatory response (DAMPs)



Autophagy/
DNA repair

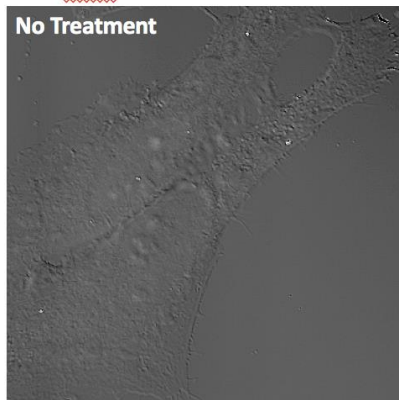
Cell Counting | Apoptosis

Active Caspase 3/7

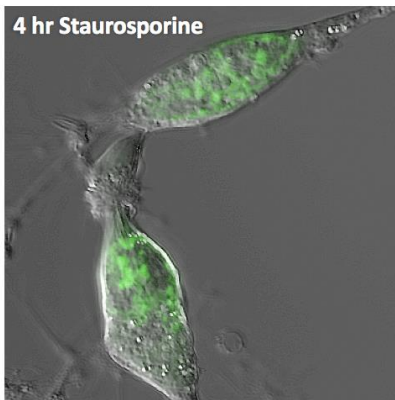


Live HeLa cells

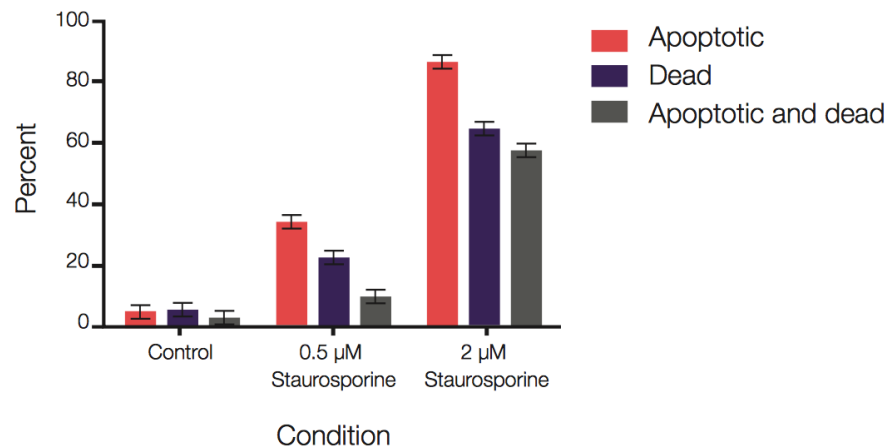
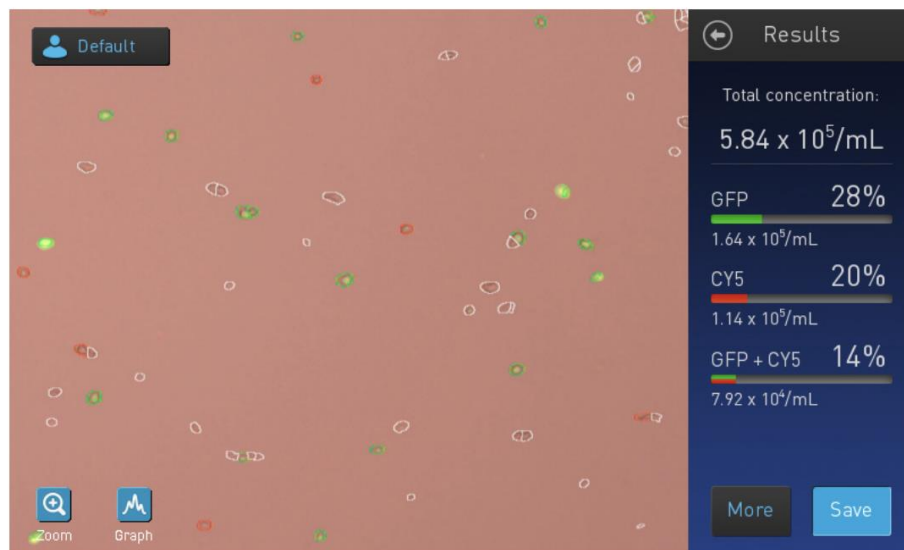
No Treatment



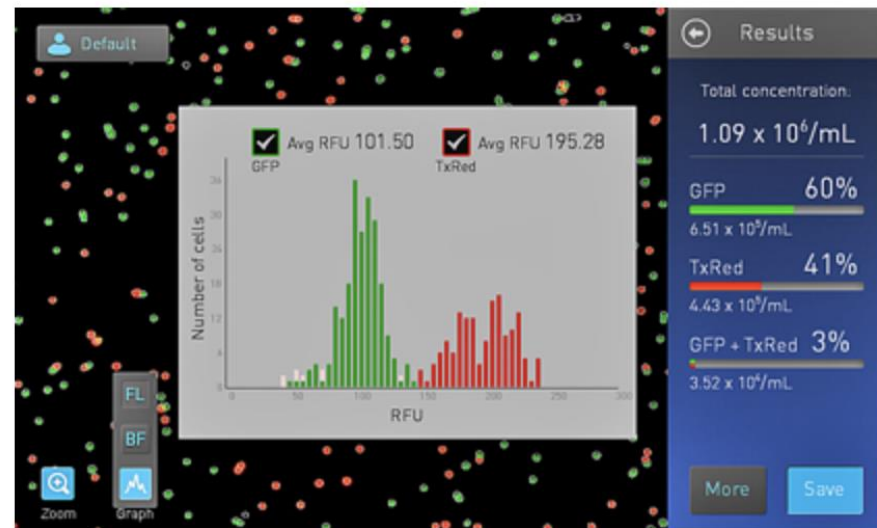
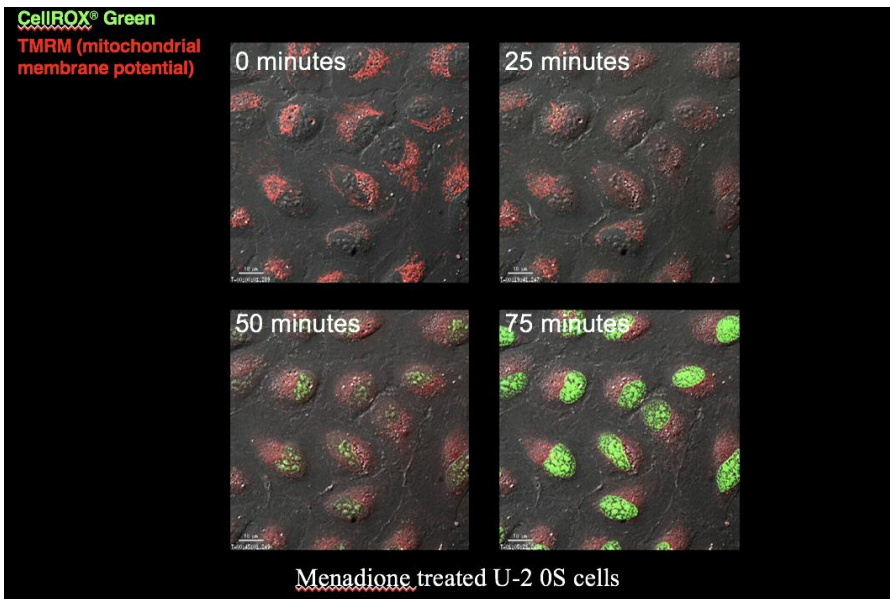
4 hr Staurosporine



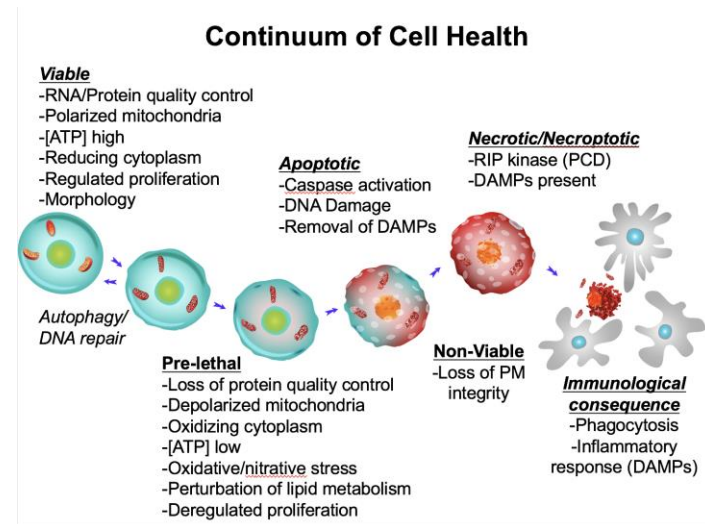
- Cells apoptotic?
- Cell Therapy – Need to know
- Untreated cells show some apoptosis
 - Normal for functional cells, but how much?



Cell Counting | Oxidative Stress & Mitochondrial Function



- Reagents for Pre Lethal readouts
- Combine in counting with gating for desired assay?
- Control population with User defined protocol



Cell Counting | Major challenges and recommendations

- Sample prep and user variability
- Cell type and form (Spheroids/Organoids) constantly changing
 - Best way to count for desired outcome difficult to predict
- Counting platforms becoming more of hybrid
 - Imaging
 - Flow
 - Classic counter
- When using limited sample how representative of entire population?
 - User training and developer agility necessary
 - Needs/Wants make it hard to establish standard across uses
 - Adaptability of instrument and user saved protocols help
- Continuum of Cell Health has to be considered with counting needs
 - Cell Therapy will need more than cell # and whether Live/Dead