Refinement of Predictive Cytotoxicity Assays Using HCA and Extension into Different Cell Lines

HCA for Toxicity Profiling, 15th Jan 2010
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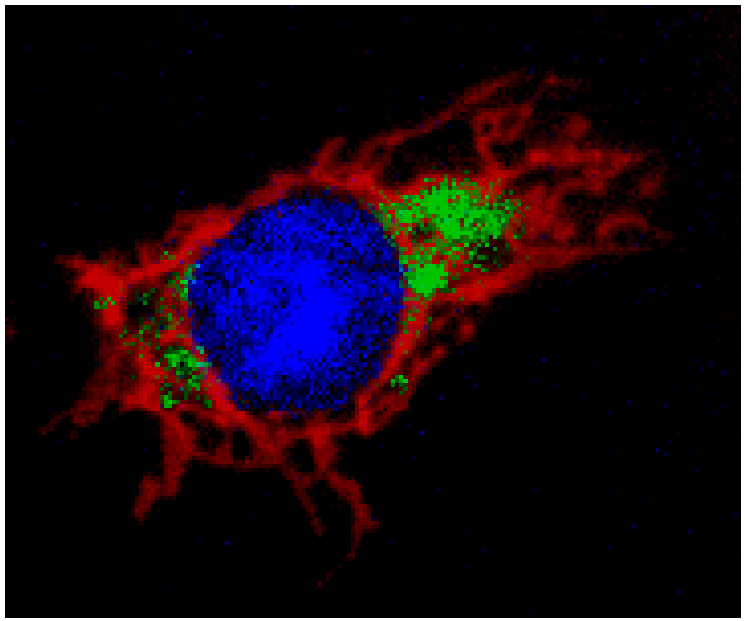
Peter O'Brien
DVM, PhD, DVSc, Dip ACVP, ECVCP, FRCPath
Conway Fellow, Director of UCD HCA Facility
Clinical Pathologist, Toxicologist
Director ADL (Advanced Diagnostics Lab)

Pathology Department
Veterinary Sciences Centre, UCD
Tel: 01716 6048
Email: Peter.James.OBrien @UCD.ie



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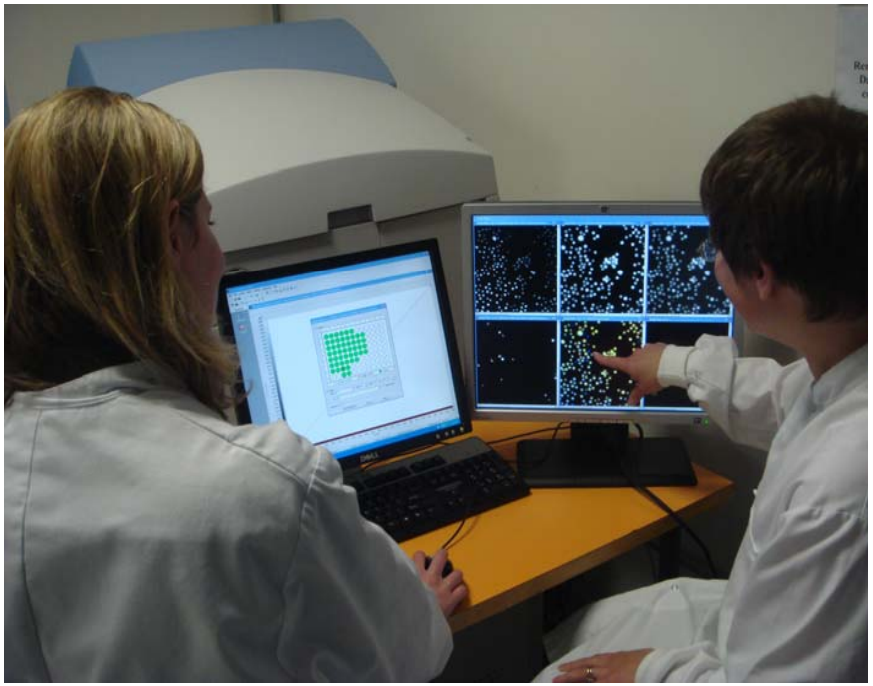

HCA Cytotoxicity Testing Shown Better Correlated with Human Hepatotoxicity than Conventional Assays or Animal Testing

	Sensitivity	Specificity
DNA synthesis	10 %	92 %
Protein synthesis	4	97
Glutathione depletion	19	85
Superoxide induction	1	97
Caspase - 3 induction	5	95
Membrane integrity	2	99
Cell viability	10	92
Cell viability or GSH or DNA Syn	25	~90
Regulatory animal toxicity tests	52	-
Cellomics Assay	**93**	**97**

O'Brien PJ, Irwin W, Diaz D, Howard-Cofield E, Krejsa CM, Slaughter MR, Gao B, Kaludercic N, Angeline A, Bernardi P, Brain P, Hougham C. High concordance of drug-induced human hepatotoxicity with in vitro cytotoxicity measured in a novel cell-based model using high content screening. Archives Toxicology Sep;80 (9):580-604, 2006.

Summary of HCA Cytotoxicity Screening Human Toxicity Potential

- Based on HCA cytotoxicity assay of 250 drugs
- Multi-parametric, live-cell, sublethal HCS cytotoxicity assay concordant with human toxicity
- Uses epifluorescence, image analysis, HepG2 in 96-well plates, dyes for: Ca, mito membrane potential, DNA, cell permeability
- Preincubate cells & drug 3d @ up to 100 Cmax
- Same specificity as past assays but much higher sensitivity than past cytotoxicity assays or even regulatory animal studies

Studies Supporting HCA Multiparametric, Cytotox Test to Assess Human Toxicity Potential

1. Noora F, Niklas J, Müller-Vieira U, Heinzle E. An integrated approach to improved toxicity prediction for the safety assessment during preclinical drug development using Hep G2 cells. Tox Appl Pharm 237: 221-31, 2009. HepG2 more effective than primary rat hepatic
2. Nogueira RC, Oliveira-Costa JF, de Sá MS, dos Santos RR, Soares MB. Early toxicity screening and selection of lead compounds for parasitic diseases. Cur Drug Targets. 10:291-8, 2009. Literature review supporting implementation
3. Abraham VC. Application of a high-content multiparameter cytotoxicity assay to prioritize compounds based on toxicity potential in humans. J Biomolec Screening, 13:527-37, 2008.
4. Xu JJ, Henstock PV, Dunn MC, Smith AR, Chabot JR, de Graaf D. Cellular imaging predictions of clinical drug-induced liver injury. Tox Sci 105:97–105, 2008. 300 drugs and chemicals; primary human hepatocytes, ~55% sensitivity, >95% specificity.
5. Jan E, Byrne SJ, Cuddihy, Davies AM, Volkov Y, Gun'ko YK, Kotov NA. High-content screening as a universal tool for fingerprinting of cytotoxicity of nanoparticles. ACS Nano, 2 928–38, 2008.
6. Schoonen, WGEJ, Westerinka WMA, de Roosa JADM, Débiton E. Cytotoxic effects of 110 reference compounds on HepG2 cells and for 60 compounds on HeLa, ECC-1 and CHO cells. I Mechanistic assays on ROS, glutathione depletion and calcein uptake. Tox in Vitro 19:491-503, 2005. 75% sensitivity

7. Schoonen, WGEJ, de Roosa JADM, Westerinka WMA, Débiton E. Cytotoxic effects of 110 reference compounds on HepG2 cells and for 60 compounds on HeLa, ECC-1 and CHO cells. II Mechanistic assays on NAD(P)H, ATP and DNA contents. Tox in Vitro 19:491-503, 2005. multiparametric biochemical endpoints in HepG2, 60 references compounds, 75% sensitivity
8. Abraham VC. Samson B, Lapets O, Haskins JR. Large-scale investigation of cytotoxicity enabled by automated simultaneous monitoring of multiple intracellular processes in individual living cells. Poster Presentation, ThermoFisher Cellomics 2003. (+ >10 others)
9. Lucke J, Mumtsidu E. A multiparametric live-cell cytotoxicity analysis using the Operetta. Application Note, Perkin Elmer. 2009.
10. Cuy JL, Ball AJ, Ryan RL, Harris DW, Hsu M, Kenneth A. Giuliano, Irwin W, Johnston PA, Taylor DL. CellCiphr™ – cellular systems biology assay for human hepatotoxicity. Poster presentation. Millipore – Cellumen 2007
11. Anderl JL, Giuliano KA, Hsu M, TaylorDL, Ball AJ. High content screening assay for hepatotoxicity using human HepG2 cells. Poster presentation, Millipore 2009.

Studies Supporting HCA Approach to Cytotox……

12. Bolt HM, Hengstler EJG. Most cited articles in the Archives of Toxicology: the debate about possibilities and limitations of in vitro toxicity tests and replacement of in vivo studies. Arch Toxicol 82:881–3, 2008. good validation study; 50 citations in literature
13. Combes RD. Some thoughts on the use of replacement alternatives for toxicity testing and risk assessment. Arch Toxicol (2009) 83:199–201. Commentary – need for validation across labs and over time

14. Hancock S, Williams A, Tinkler H, Davies I, Ismail R, Stubbs S, Thomas N, Norey C. Extracting high content data from a human hepatotoxicity screen on IN Cell Analyzer using IN Cell Investigator Poster Presentation, GE HealthCare Soc Biomolec Sci SBS 15th Ann Conf& Exh 2009, Lille, France.

What is Experimental Approach?

- 96-well, high-quality (Matriplate™) polylysine-coated plates
- Seed at 9000 cells / well
- Load 7 drugs per plate, 12 conc / per drug, up to 100 uM, or 100 x Cmax, conc serially diluted in steps of 3-fold
- Postive (FCCP for mitochondria, Triton X for membrane permeability, ionomycin for Ca) and negative controls (no treatment)
- After 3 d incubation, load plate with cocktail of dyes for 1h @ 37°C and measure fluorescence versus drug conc
- Look at dose-response curve for concentration where signal changes
- ANOVA comparison of signals at each conc to baseline

Goals of Study

- That HCA assay reported from Pfizer translates to different a) geographic locations, b) HCA technologies*
- Validate for application in additional cell lines
- Optimise for quality & ease using commercially-available biological reagents* & high-quality plates*
- Assess HCA biomarkers for translation from *in vitro* HepG2 model to *in vivo* use for assessment of toxicity

*GE HealthCare InCell 1000, Cell Factory HepG2, & Matriplate microplates

Biomarker Dyes

- Hoechst 33342 (0.8 µM) – cell number, nuclei intensity and area
- Fluo 4-AM (1µM) – intracellular Ca^{2+}
- TMRM (20 nM) – mitochondrial membrane potential
- TOTO 3 (1 µM) – cell membrane permeability

Novel Features (vs 2006 report)

- use of quality-managed, commercial biological reagents (Cell Factory™, GE Healthcare) to improve precision and reduce labour intensiveness
- use high-quality (Matriplate™, GE Healthcare) micro-plates to reduce repeat frequency & increase data quality
- higher seeding density: 9000 cells / well (vs 5000)
- serial dilution of drug in steps of 3-fold rather than 2-fold to cover a wider range of concentrations
- Use of In Cell 1000, GE Healthcare (used Cellomics KSR)

Done at UCD and ADL in Dublin (formerly at Pfizer)

O'Brien PJ. High content analysis of sublethal cytotoxicity in human HepG2 hepatocytes for assessing potential and mechanism for chemical and drug-induced human toxicity. In High content screening science, techniques and applications. Haney, SA. (ed.) Feb, 2008. 424 pp

Optimising Biological Reagents for Quality and Ease of Use

Why?: Largest source of imprecision is in cell viability which affects cell response assessment.

One of most labour-intensive and costly parts of testing is maintaining and preparing cells for use.

Criteria of optimisation:

- Highly standardized cell-handling so high imprecision
- The right cell type – HepG2 for now, downstream others (cardiac, renal, neuronal, lymphoid)
- Large-scale for access and precision over time
- Quality-managed
- End-user ease of use: “thaw and seed”
- Cost-effective

Solution for study: GE Healthcare Cell factory HepG2 cells

Optimising Plates

Need High Optical-Quality microplates

- glass-bottomed
- Uniform thickness
- thin
- Well bottom flatness
- low background fluorescence
- no crosstalk

We used MatriPlates™ with the thin bottom (0.17 mm)

Characterisation of Imprecision

Protocol - based on international consensus protocol of CLSI (Clinical Laboratory Standards Institute)

- assessed over 5 days with positive and negative controls
- assess as cv (%) ie SD / Mean
- assessed across plates, wells, fields, cells, 2 analysers

	Plate to Plate	Well to Well	Field to Field	Cell to Cell
Nuclear area	3.0	2.9	6.9	39.2
Nuclear intensity	10.7	2.4	7.3	38.2
Mito mem potential	2.4	2.1	5.9	20.7
Ionised Ca	9.2	4.7	3.0	10.2
Plasma mem perm	6.2	5.4	5.2	36.1
Cell number	10.5	14.3	44.0	N/A

Note: no significant differnces across analysers found.

Conclusions on Imprecision Studies

- Biological response is by far the greatest source of variance as indicated by cell-to-cell imprecision exceeding that across wells, fields, or plates
- Cell clumping is largest source of variance in cell number as indicated by high field-variance

	Plate to Plate	Well to Well	Field to Field	Cell to Cell
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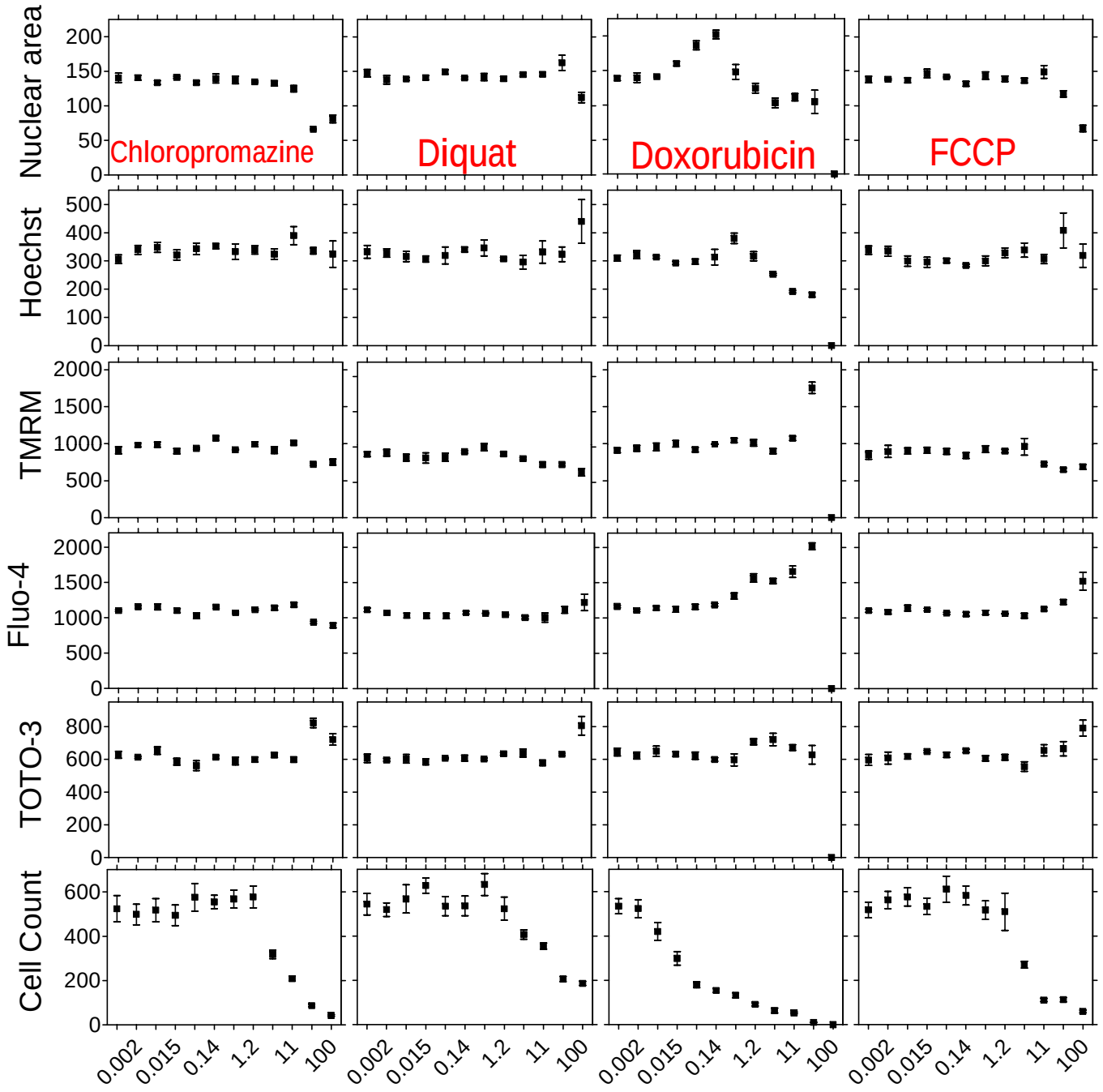
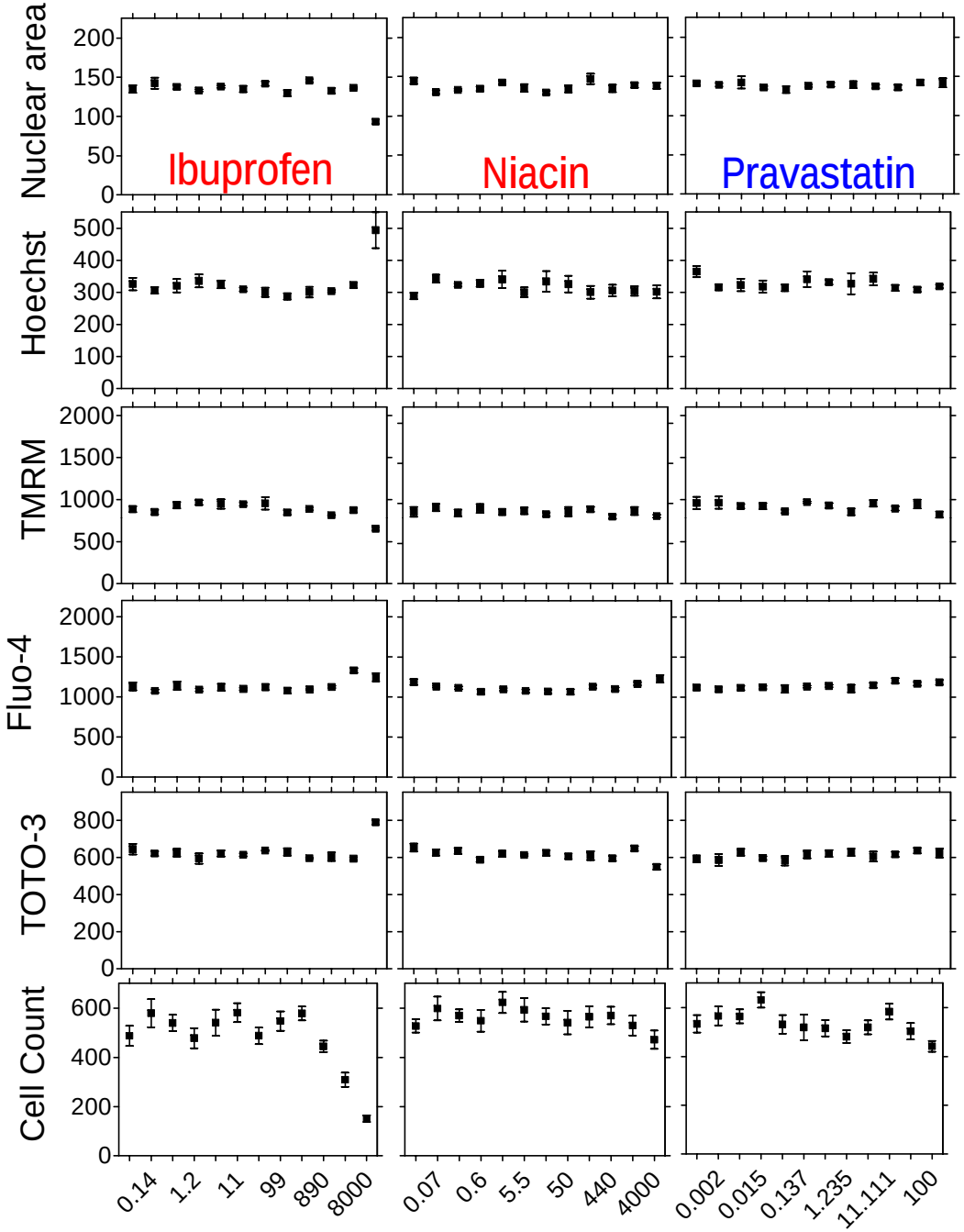


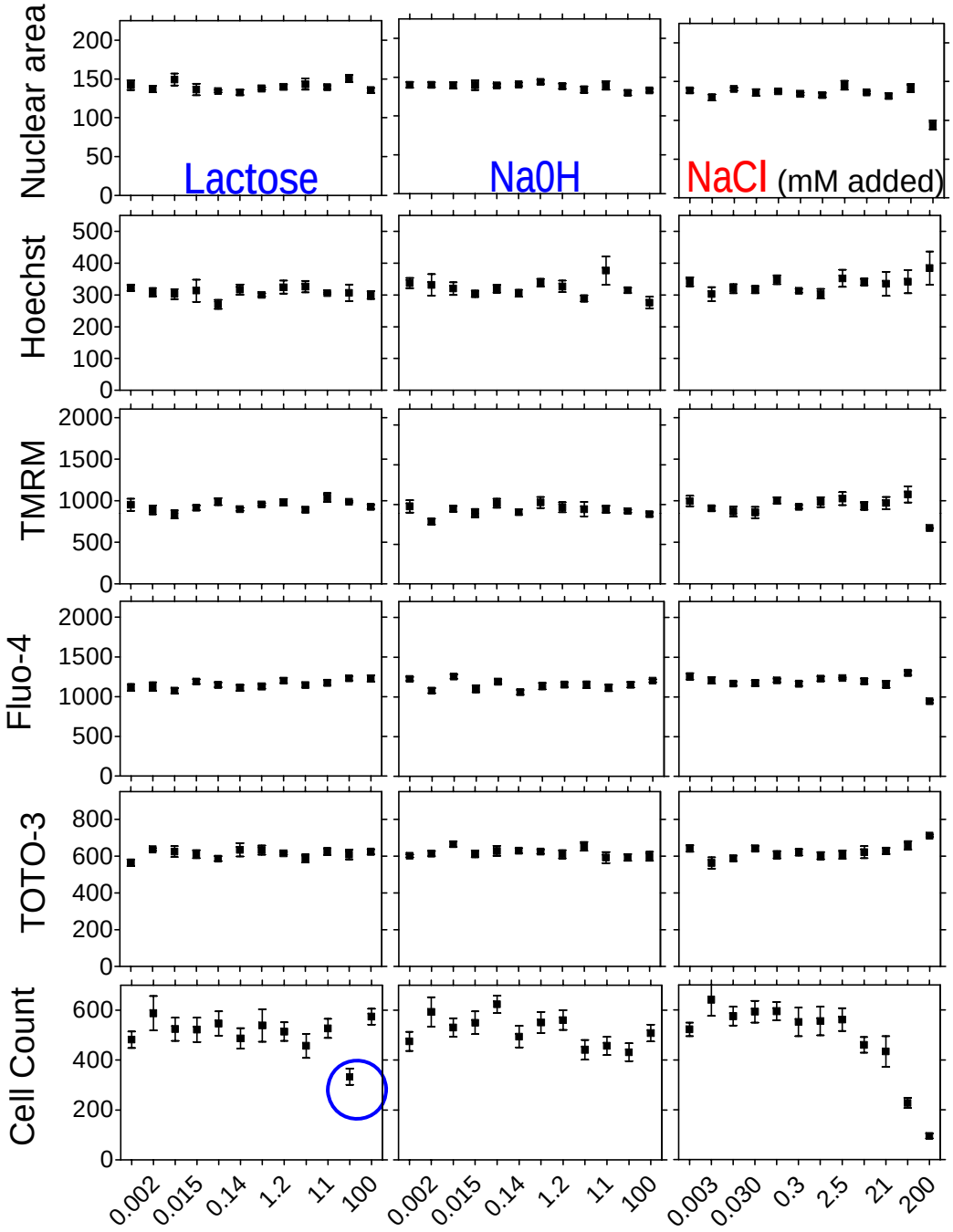
Dose Response Curves for Toxic Drugs and Chemicals

- Doxorubicin (and its congeners) is only drug to substantially lower nuclear intensity (competing with Hoechst for intercalation), show intrinsic fluorescence interfering with fluo4, and apparently affect drug exporters to increase TMRM)



Toxic versus Nontoxic Drugs and Chemicals

- Pravastatin has several hundred-fold lower toxicity than its lethal congener cerivastatin



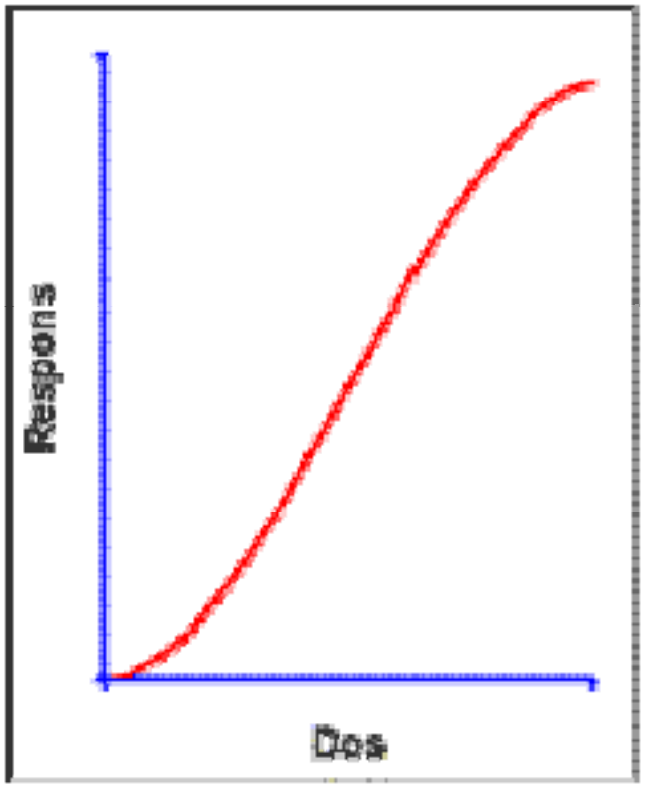
- Cell number is most effective indicator by far and away
- Hyperosmolar conditions are toxic
- Data needs to be screened for outliers
- Cells and medium can buffer 100 uM base

Outlier

Fundamental Tenet of Toxicologists attributable to

Paracelsus (1493-1541), a Swiss alchemist and physician considered to be "Herald of Modern Toxicology"

"Dosis facit venenum." "The dose makes the poison."

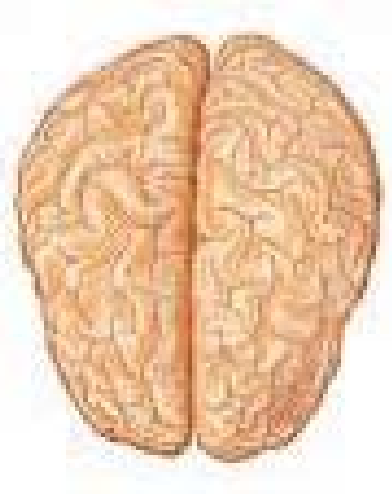


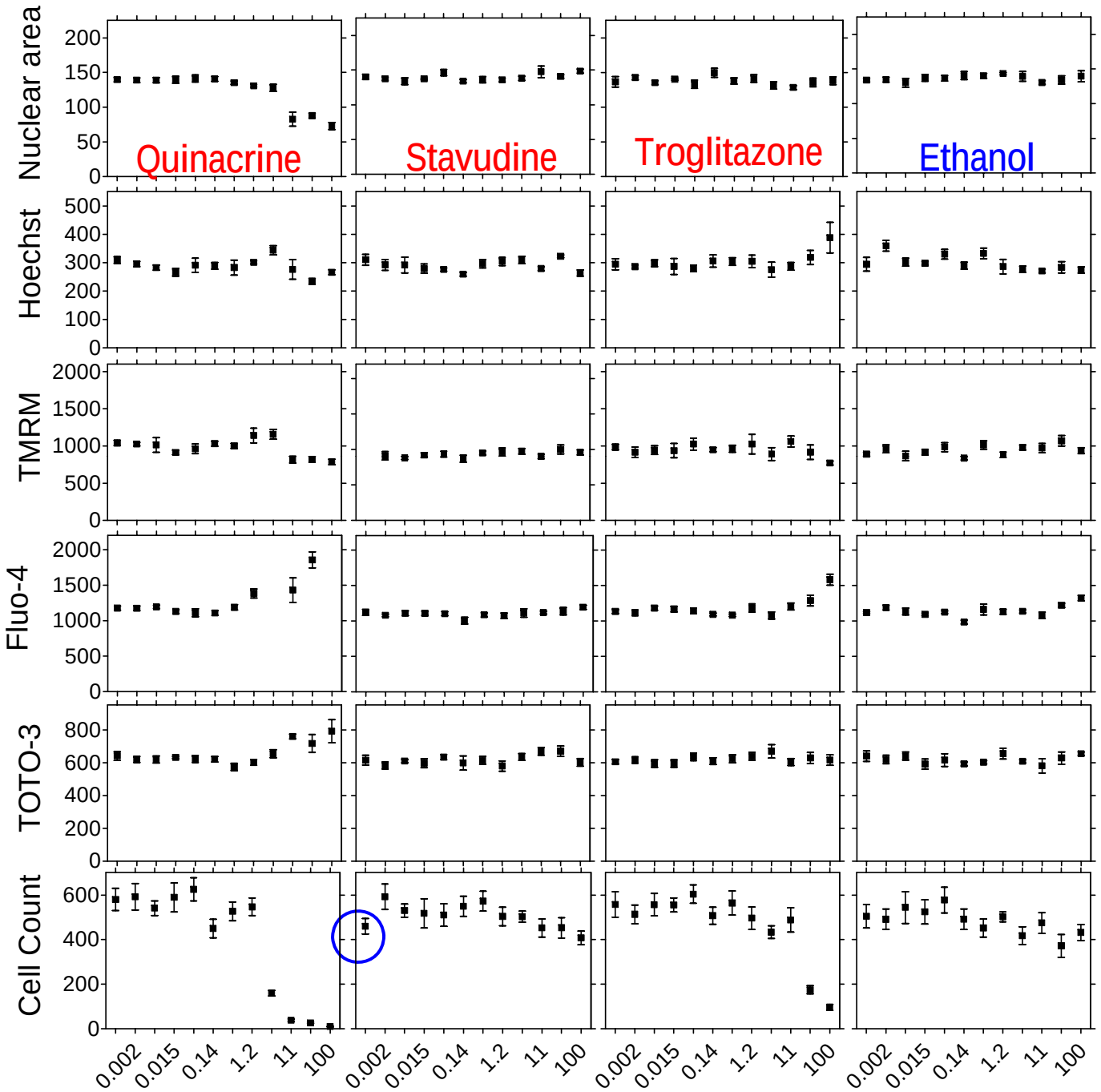
i.e. "All substances are poisons"; there is none which is not a poison, if it is bioavailable and there is sufficient exposure.

Hence we always dose until we get a toxic response.

Water Intoxication

- 300 deaths / year in US esp after excessive sweating, forced consumption
- Adult requires 1.5-2 L / d; can excrete ~20 L / day
- 3 L at a single sitting can kill
- Safety margin ~10; with toxic dose ~ 40 g/kg i.e. ~3 kg
- Death from hyperhydration: hyponatremia causing cerebral edema or heart failure





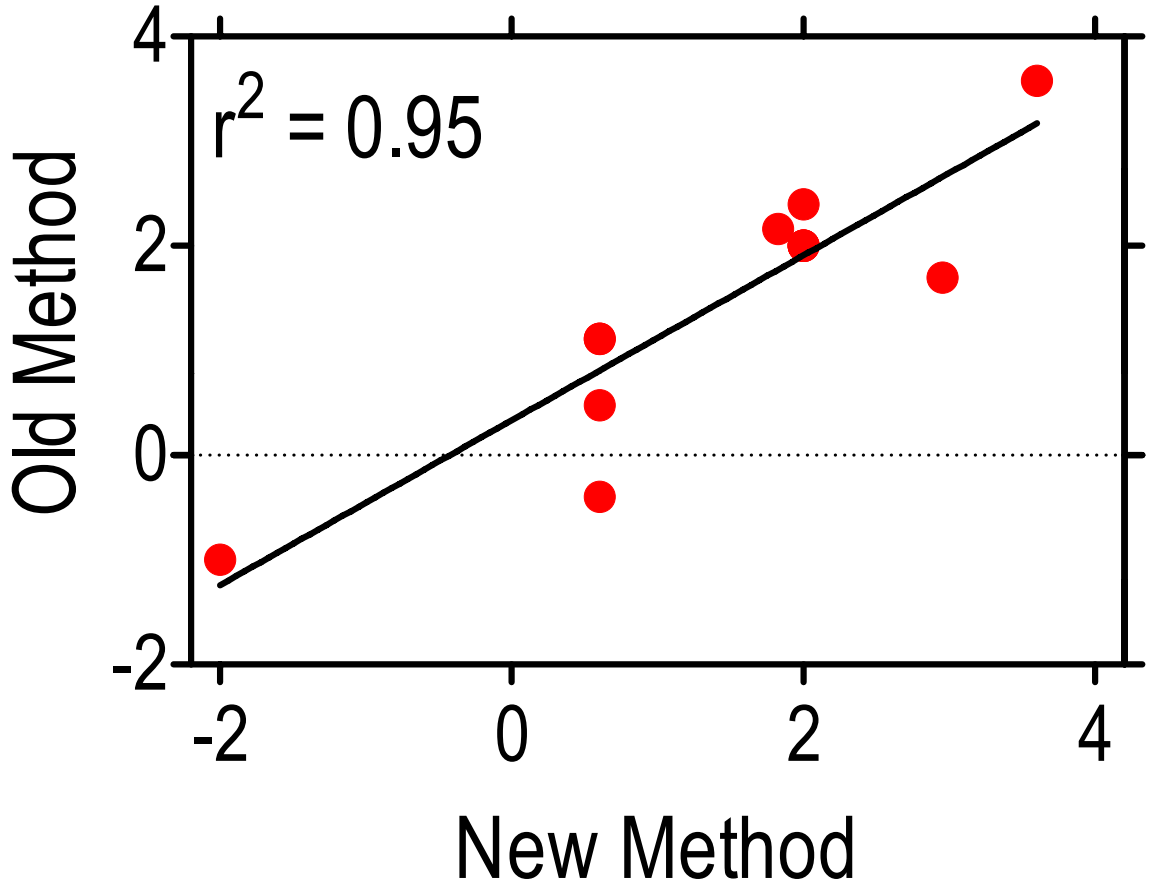
- Stavudine is a chronic toxicant because it inhibits mitochondrial DNA synthesis, a relatively slow process.
- Troglitazone stands out compared to the other glitazones for its toxicity.

○ Outlier

Method Comparison

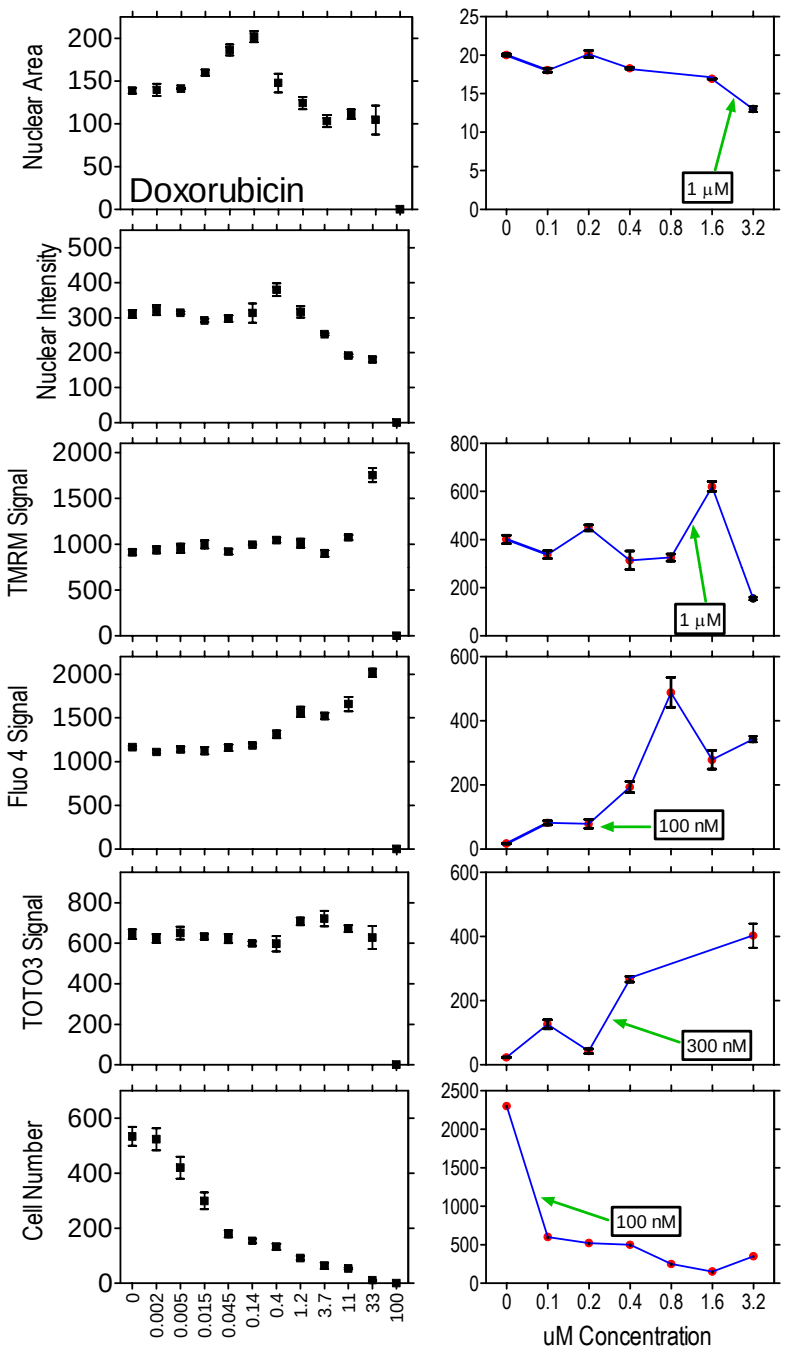
	Toxic Concentration (uM)		Cmax	TI
	New Method	Old Method (2006)		
Chlorpromazine	4	3	0.6	5
Diquat	4	13	NA	NA
Doxorubicin	0.01	<0.1	0.2	0.1
FCCP	4	13	NA	NA
Ibuprofen	900	50	250	4
Niacin	4000	3800	126	30
Pravastatin	100	100	0.1	1000
Quinacrine	4	0.4	1	2
Stavudine	>100	250	4	63
Troglitazone	33	-	5	7
Ethanol	>100	>100	NA	NA
Lactose	>100	>100	NA	NA
NaOH	>100	>100	NA	NA
NaCl	67 mM	20 mM	NA	NA

Method Comparison



All compounds were correctly identified as to their toxicity by both methods.

HCA Cytotoxicity Assay for Doxorubicin



- similar dose-response curves
- similar parameters affected
- biphasic dose reponse curves
- doxorubicin (and its congeners) is only drug to

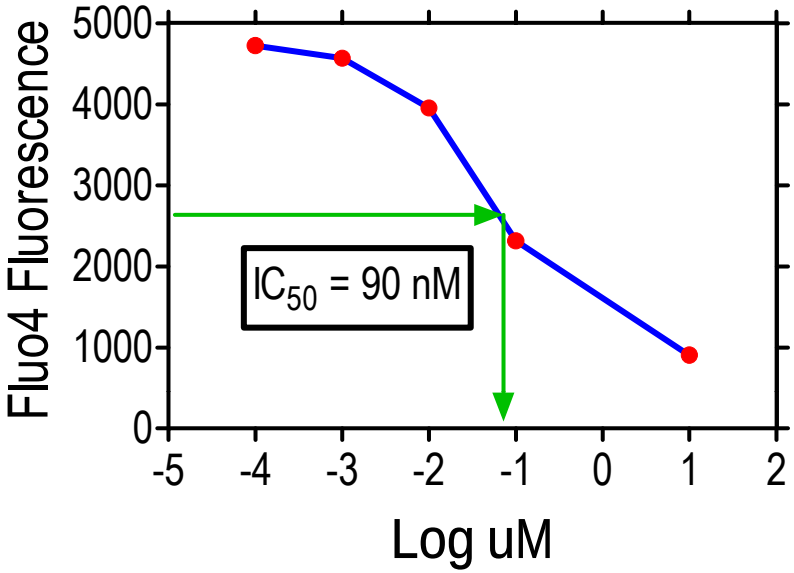
(a) substantially lower nuclear intensity by competing with Hoechst for intercalation

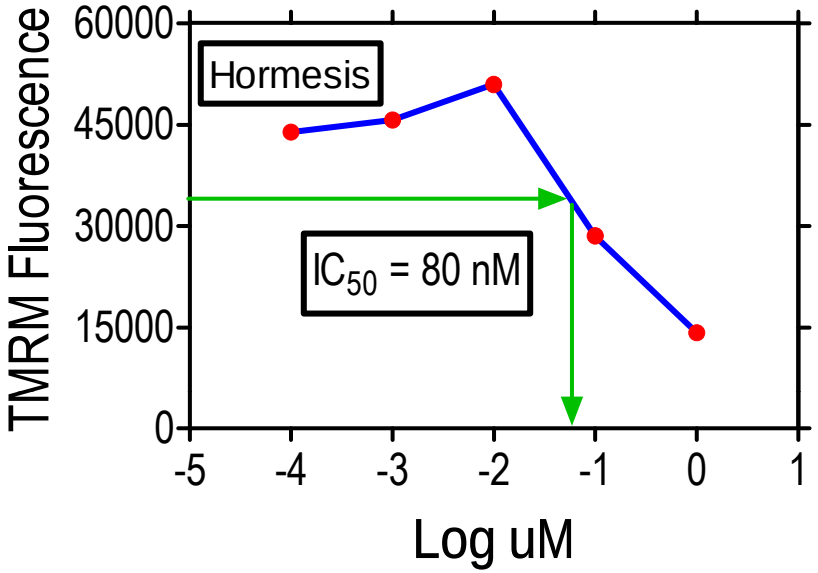
(b) show intrinsic fluorescence interfering with fluo4

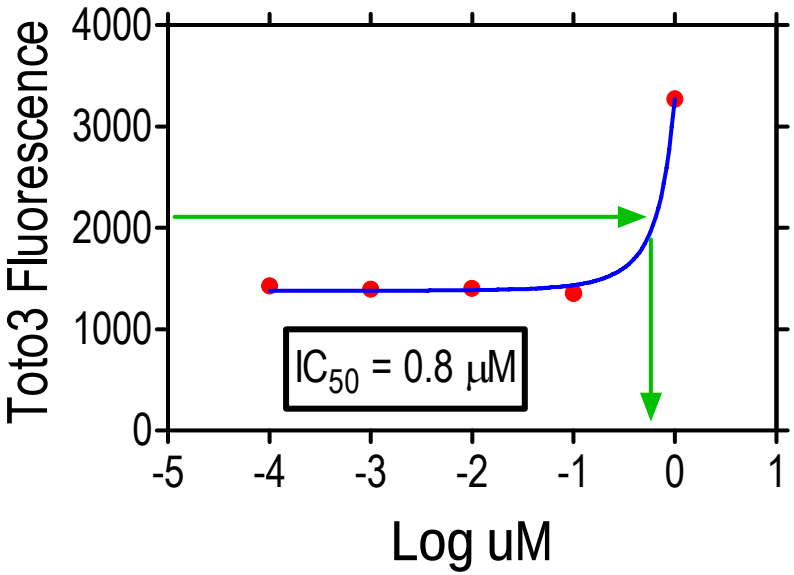
(c) apparently affect drug exporters to increase TMRM signal

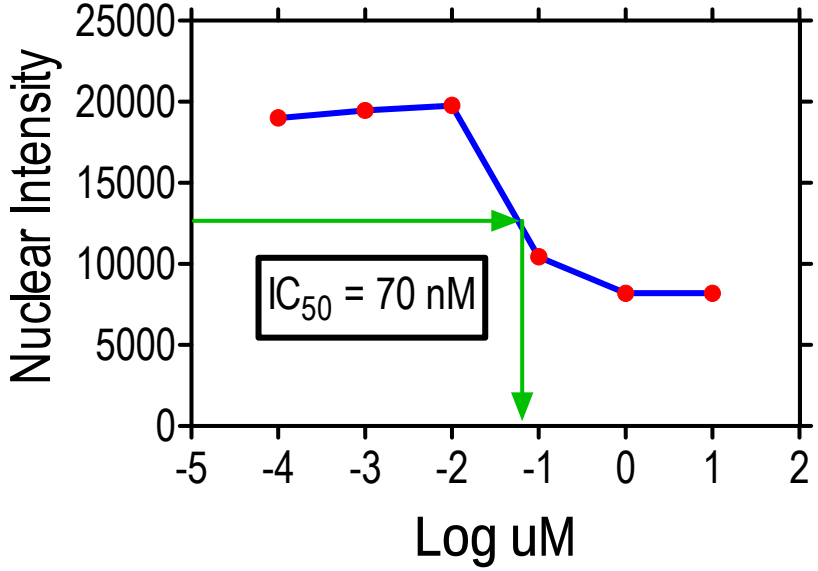
(d) Cause marked increase in nuclear area

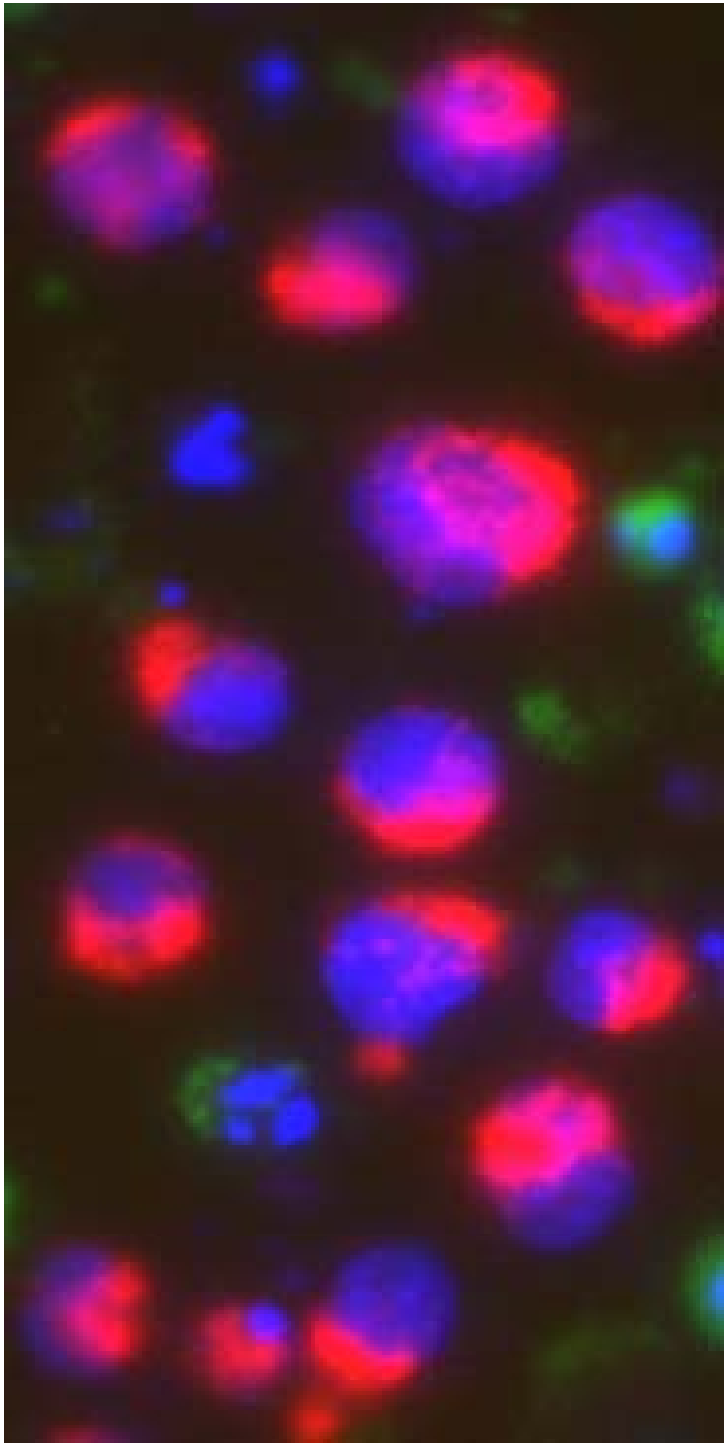
Doxorubicin-induced Lymphotoxicity











Lymphotoxicity

Toxicant

1 – 3 days

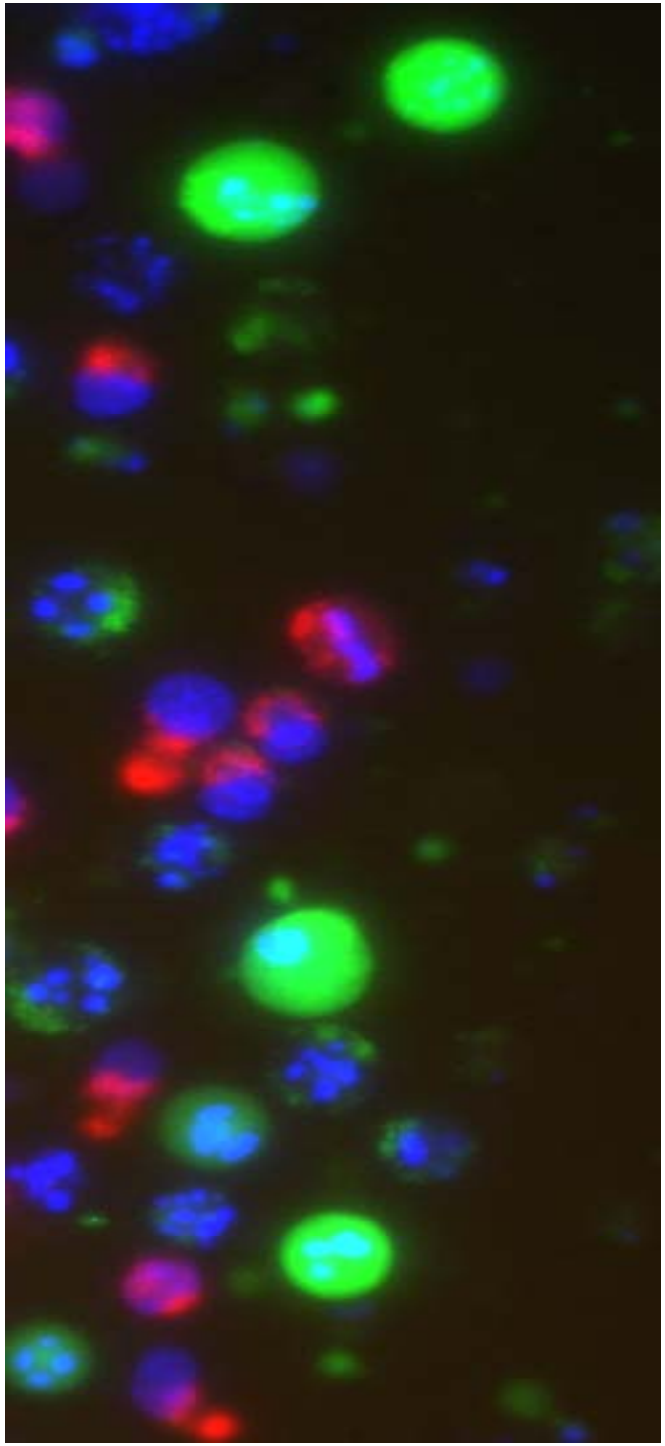
Red = TMRM

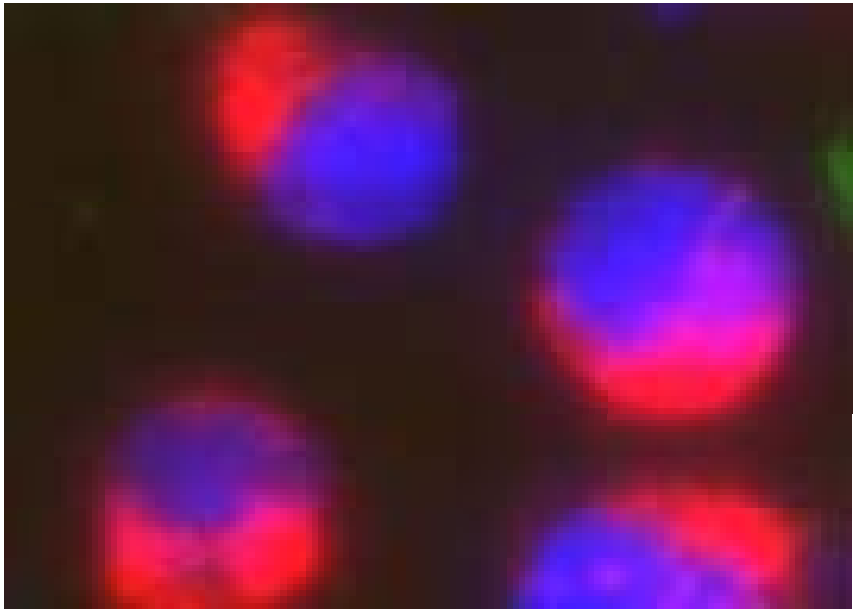
Green = Fluo 4

Blue = Nuclear DNA

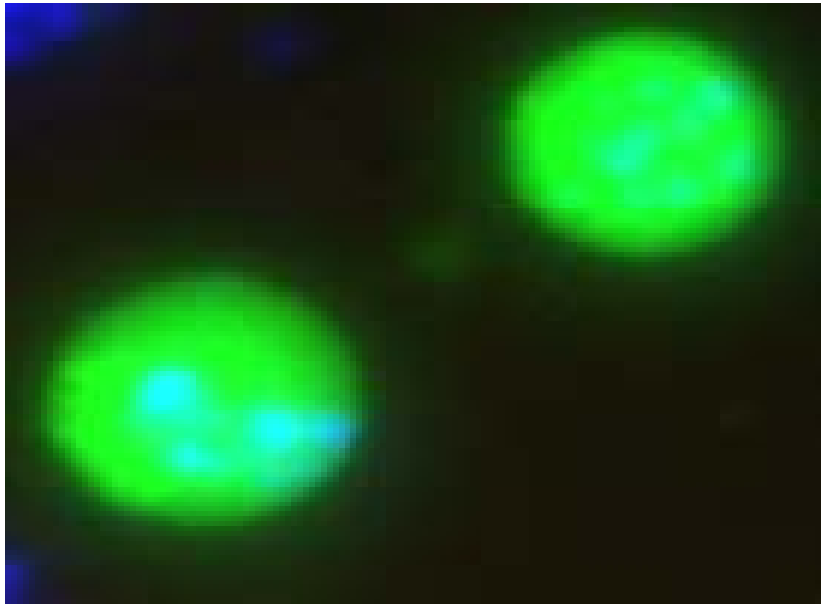
20x objective

HuT 78





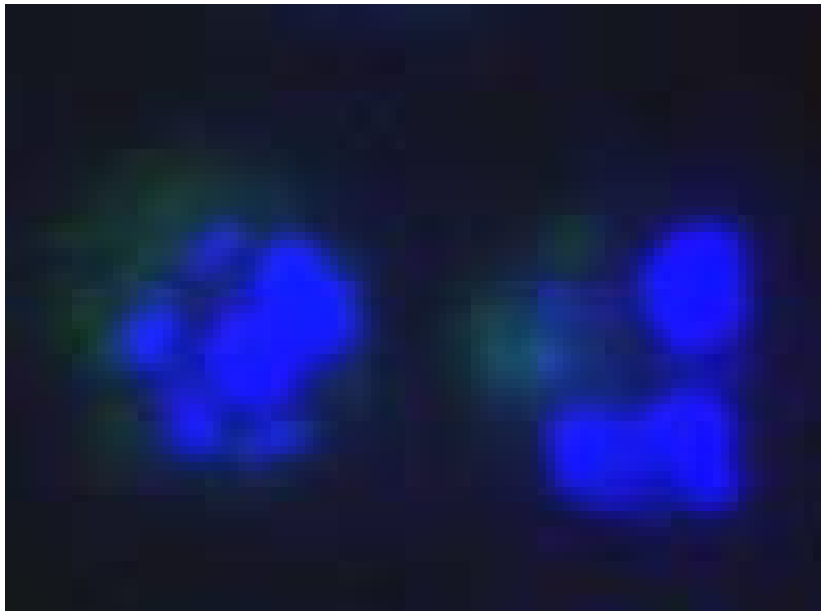
Healthy cells



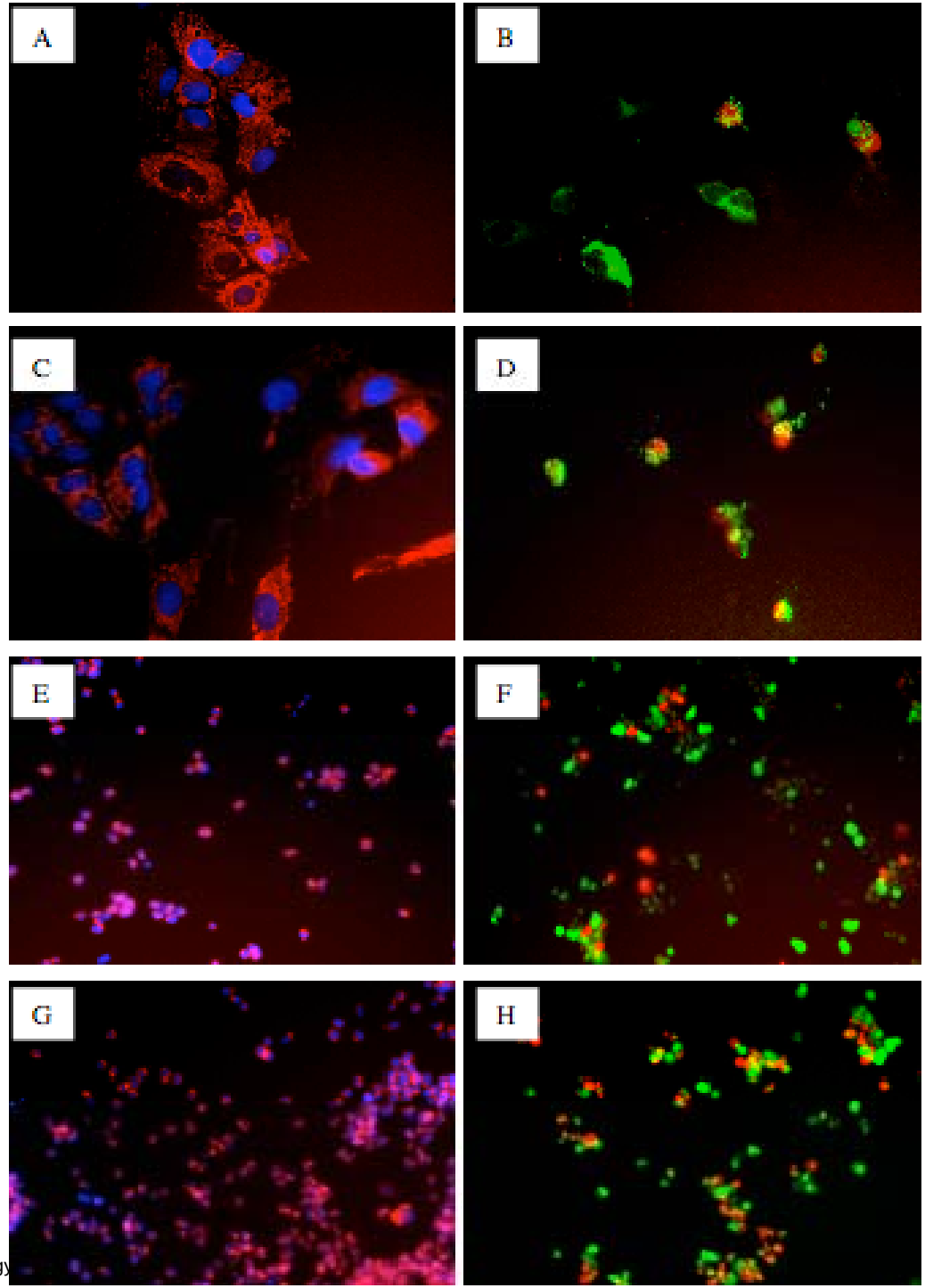
↑Ca^{2+}

Sequence of Events in Cell Death

1) Loss of mitochondrial activity
2) Increase in ionised calcium
3) Apoptosis- nuclear fragmentation
4) Loss of Ca dyes



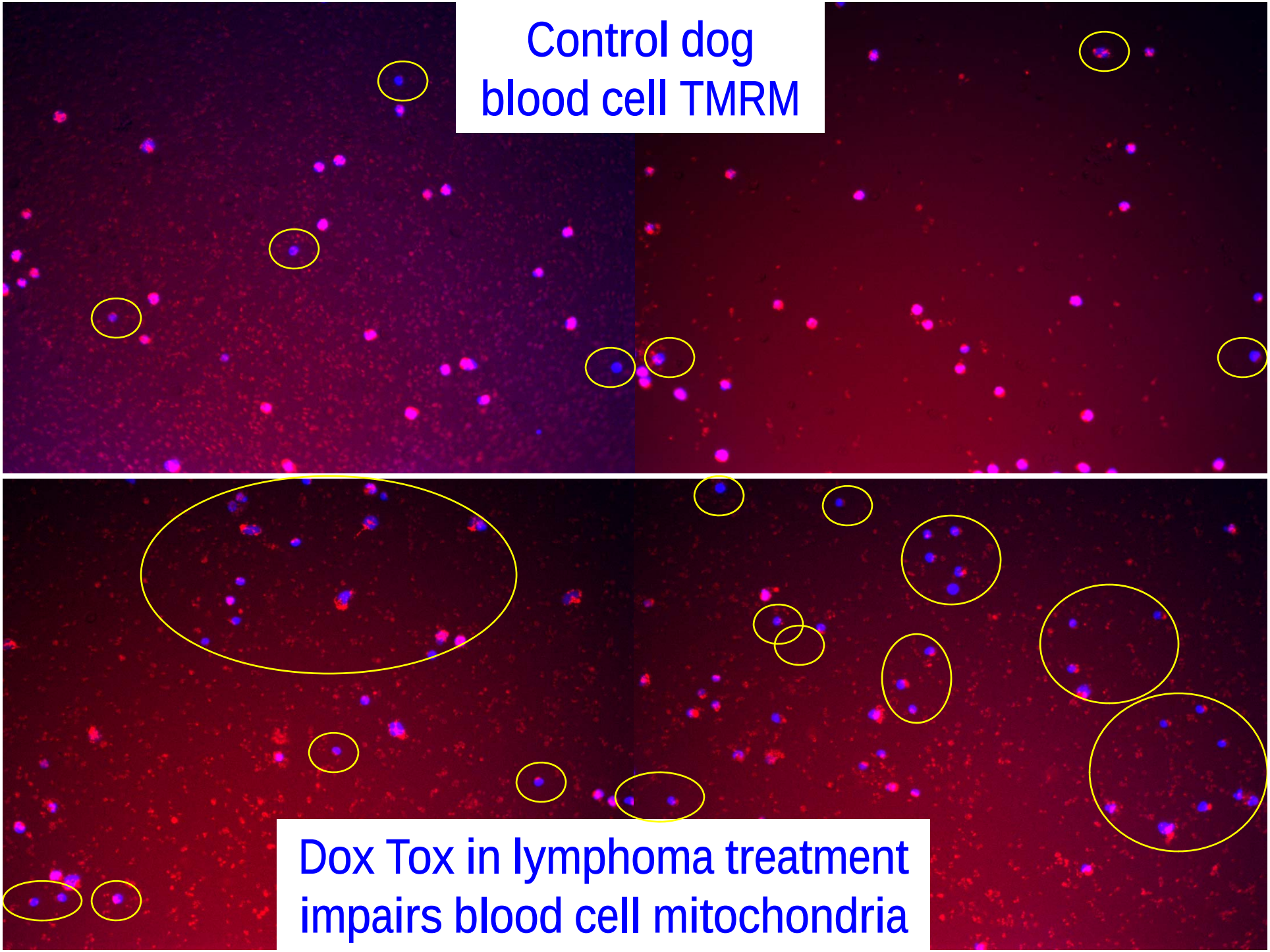
Apoptosis

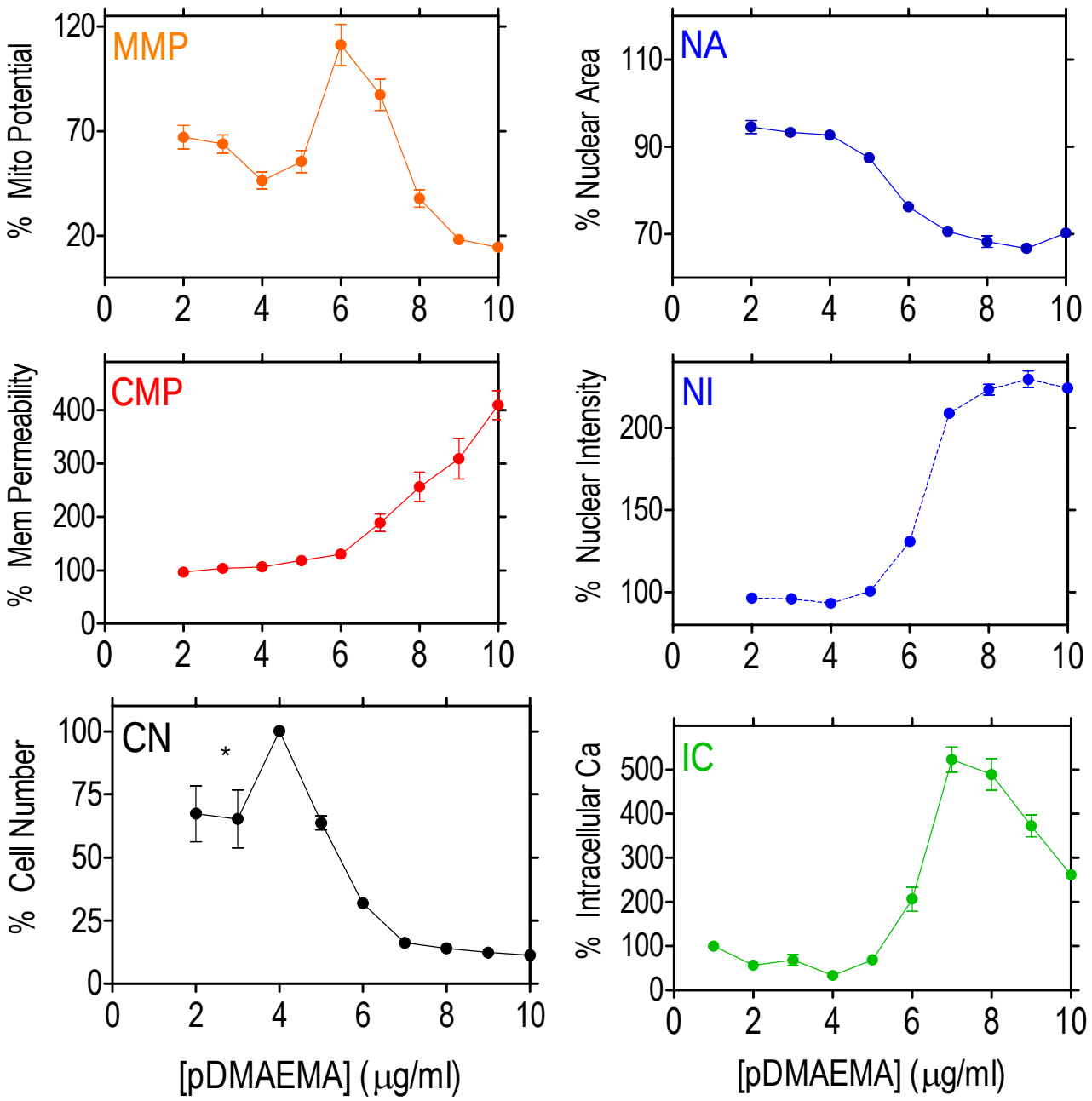


Healthy Cells versus Cytotoxic Cells

Hoechst
TMRM
Fluo-4
TOTO-3

Lieggi et al. 2009, Toxicology


Control dog
blood cell TMRM
Dox Tox in lymphoma treatment
impairs blood cell mitochondria



Dose Response Curves for Cytotoxicity of the Drug Carrier pDMAEMA

(poly [2-dimethylaminoethyl methacrylate])

in Caco-2 Cells

(human colon adenocarcinoma)

Conclusions on HCA Cytotoxicity Assay

- That HCA assay reported from Pfizer translates to different a) geographic locations, b) HCA technologies*
- Cytotox assay now validated for application in hepatocytes, myocytes (data not shown), Caco-2 intestinal cells, lymphocytes, blood cells
- Cytotox assay optimised for quality & ease using commercially-available biological reagents* & high-quality plates*
- HCA biomarkers for *in vitro* and *in vivo* anthracycline cytotoxicity as well as for quantifying drug concentration, efficacy (intercalation and Hoechst displacement)

*eg GE HealthCare InCell 1000, Cell Factory HepG2, & Matriplate microplates

ADL ADVERT

Contact: Peter.James.OBrien@UCD.ie

1) Toxicologic Clinical Pathology testing
2) Cytotoxicity Testing Service: 100 € per sample

UCD Campus Centre

NovaUCD (ADL in Lab 013)