

The Number of Genes Changing Expression after Chronic Exposure to CDMA or FDMA Radiofrequency Radiation does not Exceed the False Positive Rate.

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Goal

Measure any changes in gene expression following exposure to RF electromagnetic radiation.

SIGNALS USED

1. FDMA [Frequency Modulation]

835.620 MHz dithered ± 0.015 MHz

2. CDMA [Code Division Multiple Access]

847.740 MHz nominally spread 1.2288 MHz

"Skirts" roll off rapidly beyond band edge:

-28.3 dBc @ ± 670 kHz

-33.3 dBc @ ± 690 kHz

-48.5 dBc @ ± 750 kHz

-50.5 dBc @ ± 900 kHz

Time domain behavior complicated:

1.25 ms pulses with pseudorandom spacing

40% duty cycle, $\sigma_p/\mu_p = 0.013$

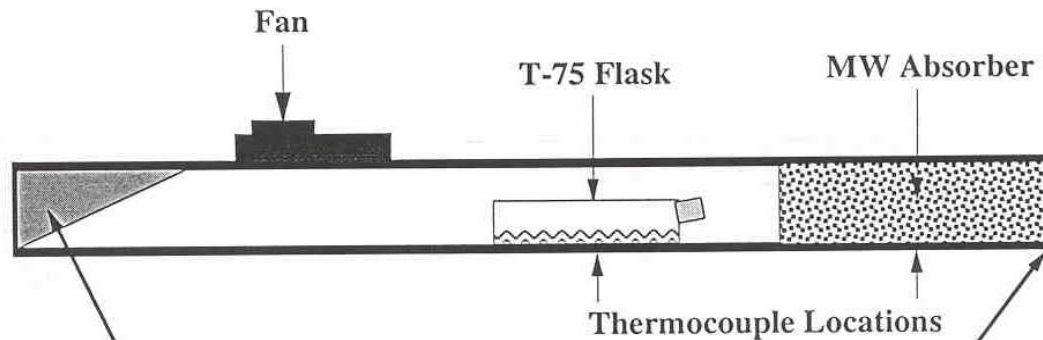
amplitude modulation of pulse

-2.5 dB to +5.1 dB

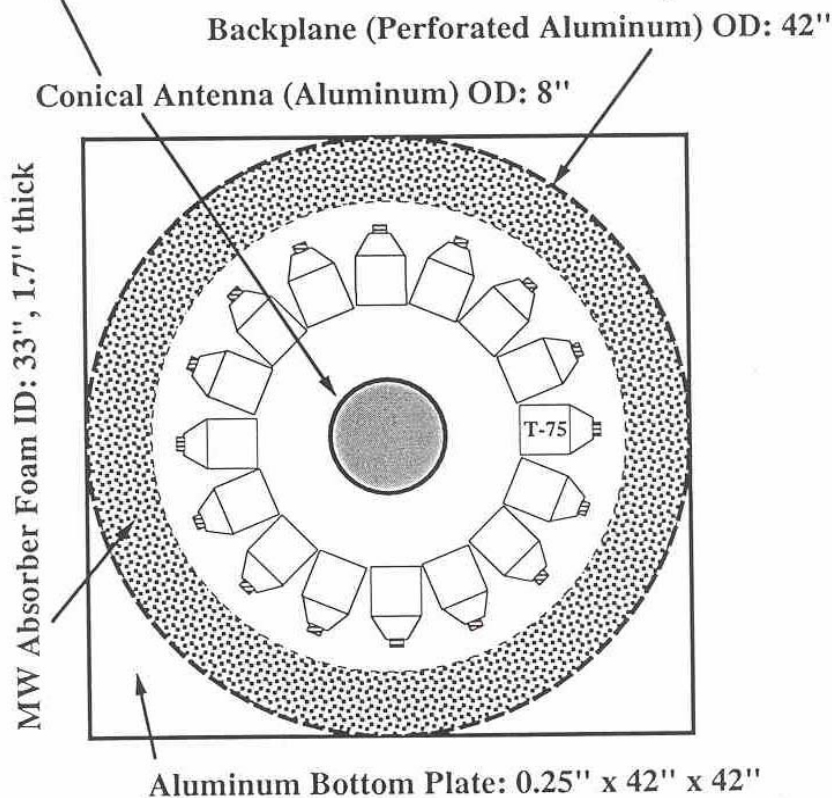
Rationale

- **Chronic exposure (24 hr) was used to look for long term changes in oncogene or tumor suppressor gene expression.**
- **The low duty cycle $\sim 40\%$ and random inter-pulse timing with frequency changes of CDMA should reduce the chances of the cells adapting to the presence of continuous energy deposition in the absence of any temperature change.**
- **FDMA provided a source of continuous energy deposition.**

RTL Interior: Side View



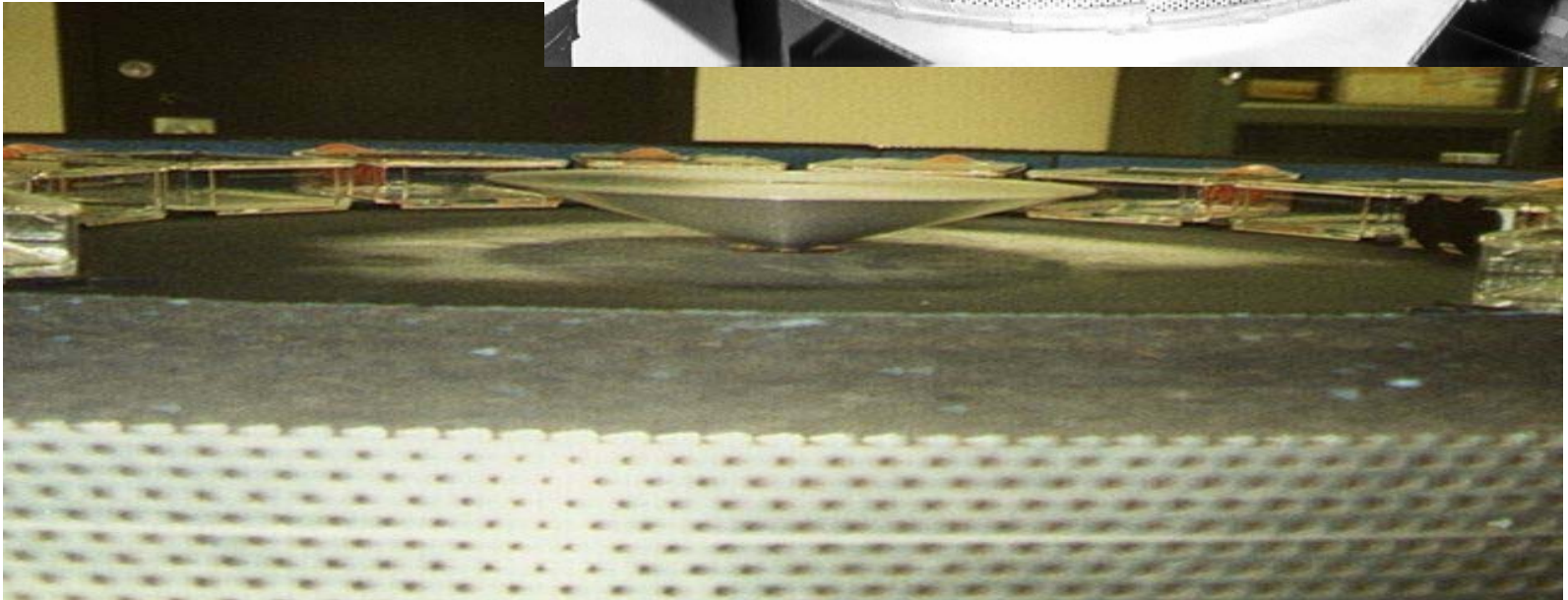
RTL Interior: Top View



In Vitro Exposure System

24 hr exposure
at ~ 5 W/Kg,
time average for
CDMA & FDMA

**RTL loaded
w
Antenna
close up**



Experimental Methods

- C3H10T1/2 cells were grown to confluence in T75 flasks with 40 ml of BME+10% FBS.
- One flask was exposed to either CDMA or FDMA RF (nominal SAR - 5 W/kg) for 24 hr, and one flask was placed in a sham RTL for 24 hr. Flasks were maintained at 37°C.
- Cells were rinsed, treated with TRIzol solution and frozen at -80°C.
- RNA isolation and Gene Chip analyses were performed by the Siteman Gene Chip Facility.

First, we determined if house keeping genes or stress response genes were changing expression due to RF exposure.

Selected ESTs present in all 12 samples, related to stress responses

Gene Symbol	CDMA				FDMA			
	treated mean	std dev	sham mean	std dev	treated mean	std dev	sham mean	std dev
Gapd	19658	4288	18859	2157	19791	3374	21151	7507
Fos	774	46	1041	89	745	388	1034	588
Jun	2287	243	2629	507	2073	912	1780	1092
Rasa3	2272	438	2206	247	1938	565	2269	725
Hsp70-2	1130	96	1036	84	1259	445	1212	434
Hsp70-3	905	164	876	94	949	39	926	41
Hsp70-4	578	31	524	42	563	47	726	84
Hsp105	2769	388	3696	430	3577	199	3658	381
Hsp110	1511	52	1343	436	1500	275	1712	230

Selected from more than thirty analyzed

Since there was no change in expression of more than thirty selected genes, we used a mathematical approach to determine the changes that were significant.

However, there will be a significant number of false positives due to multiple comparisons.

Positive and Negative Controls

- 1.** Sham versus sham provides a negative control and estimation of the false positive rate.
- 2.** We used 0.68 Gy of X-rays as positive control. Gene expression was measured 4 hr after irradiation.
- 3.** A low dose with moderately long post irradiation time was used to generate small (1.3-2 fold) changes in gene expression.

Algorithm for Determining Significant Gene Expressions I

- 1.** Two-tailed t-test was performed for each gene in \log_2 space. Fold change = $\text{mean}(\log_2[\text{treated}]) - \text{mean}(\log_2[\text{sham}])$. Those genes with $p < 0.05$ were separated into up- and down-regulated groups.

Algorithm for Determining Significant Gene Expressions II

2. Those genes with $p < 0.05$, were evaluated using the Affymetrix “Absolute Call” criterion.
 - An up-regulated gene was considered “real” if it was assessed as “present” or “marginally present” on all 3 of the RF exposed GeneChips[®].
 - A down-regulated gene was considered “real” if it was assessed as “present” or “marginally present” on all 3 of the sham exposed GeneChips[®].

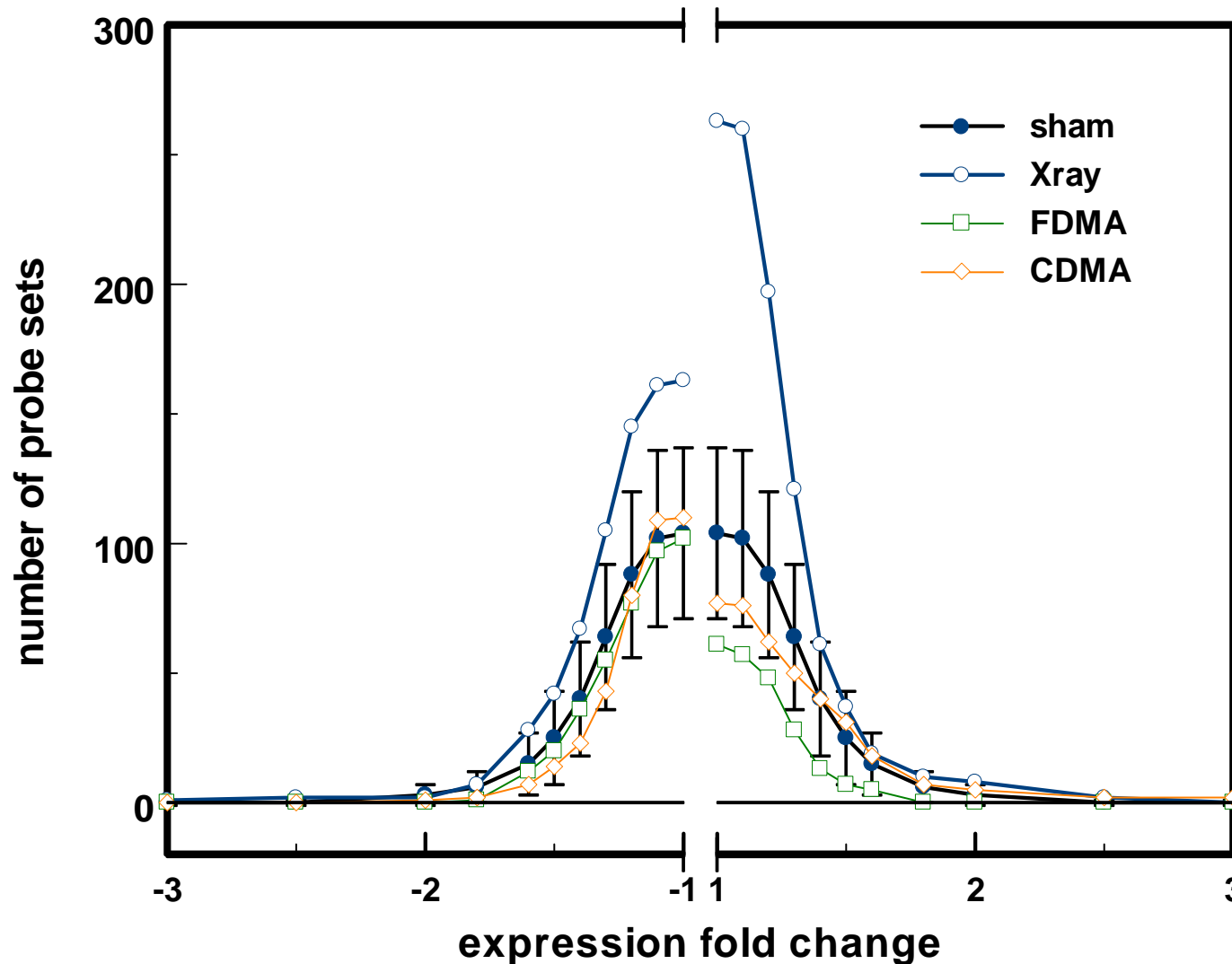
Algorithm for Determining Significant Gene Expressions

- 3.** The expected number of false positives was estimated by separating the 6 sham GeneChips[®] into 2 groups of 3, and subjecting them to the above algorithm. All 20 permutations were evaluated and the mean number of false positives and standard deviations were calculated at various fold changes.

Algorithm for Determining Significant Gene Expressions

4. If the number of positives identified from treated vs. sham analysis is $<$ the number of expected false positives, then it is likely that those expression changes are false positives.
 - Only if the number of positives exceeds the expected number of false positives is further investigation warranted.

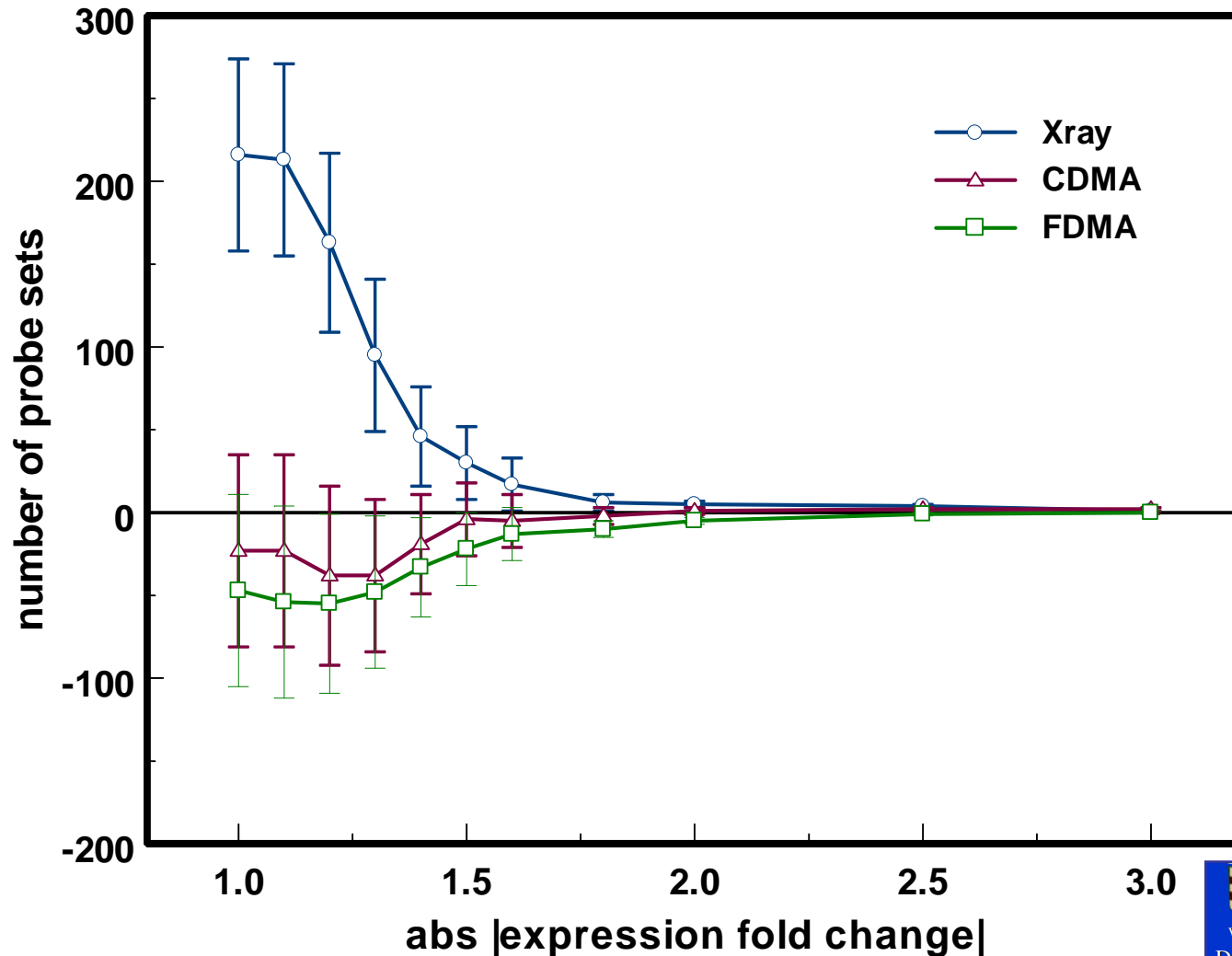
Number of Probe Sets with a Statistically Significant Change in Expression



Summary

- 1.** In the sham versus sham analysis the number of up-regulated probes was approximately equal to the number of down-regulated probes, consistent with both being random.
- 2.** The number of up-regulated probes was greater than down-regulated consistent with a non-random effect.
- 3.** The numbers for up- or down-regulated probes after RF was lower than in the sham versus sham analysis, as illustrated in the next two slides.

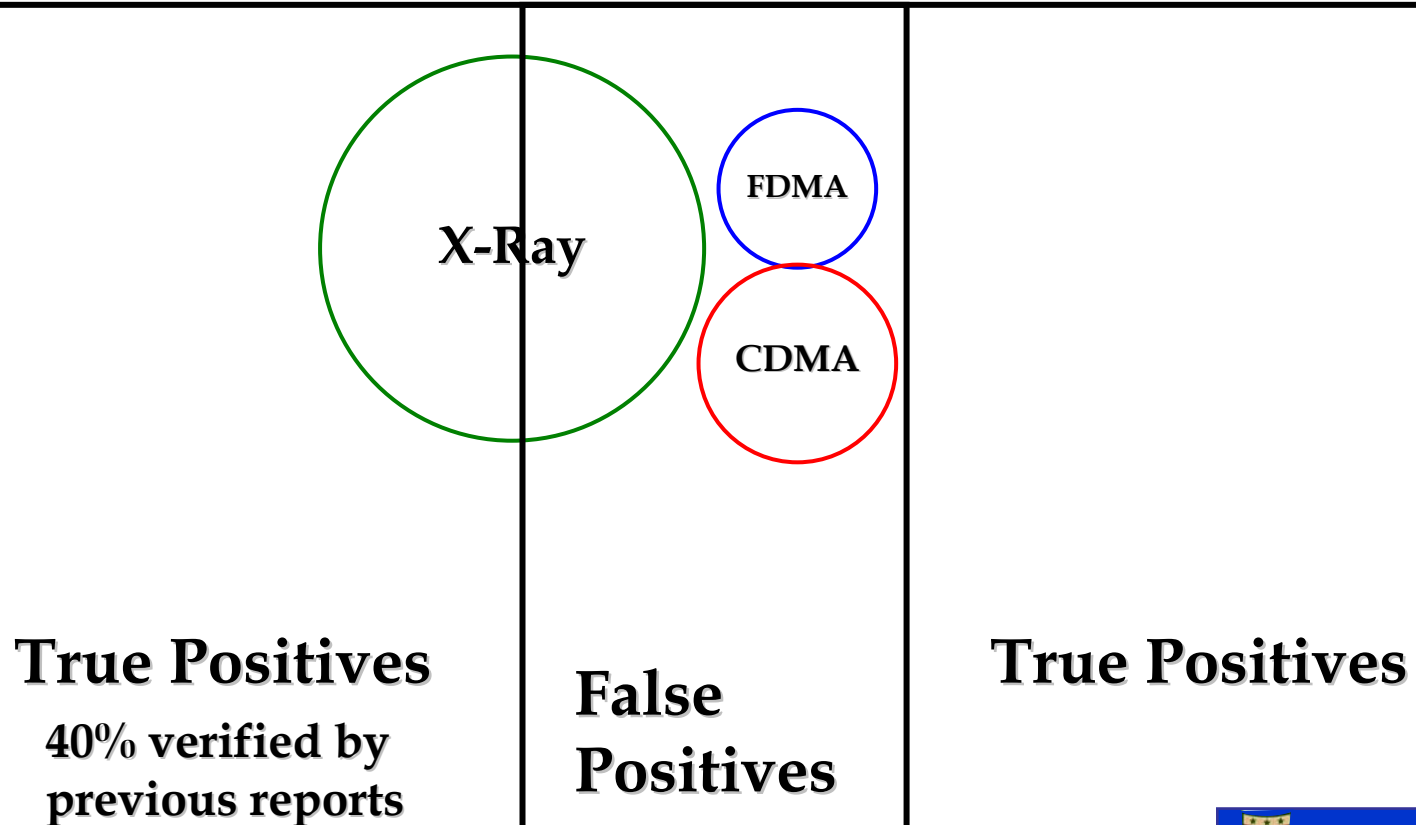
Number of Probe Sets with a Statistically Significant, Absolute Change in Expression Relative to the Sham versus Sham Number



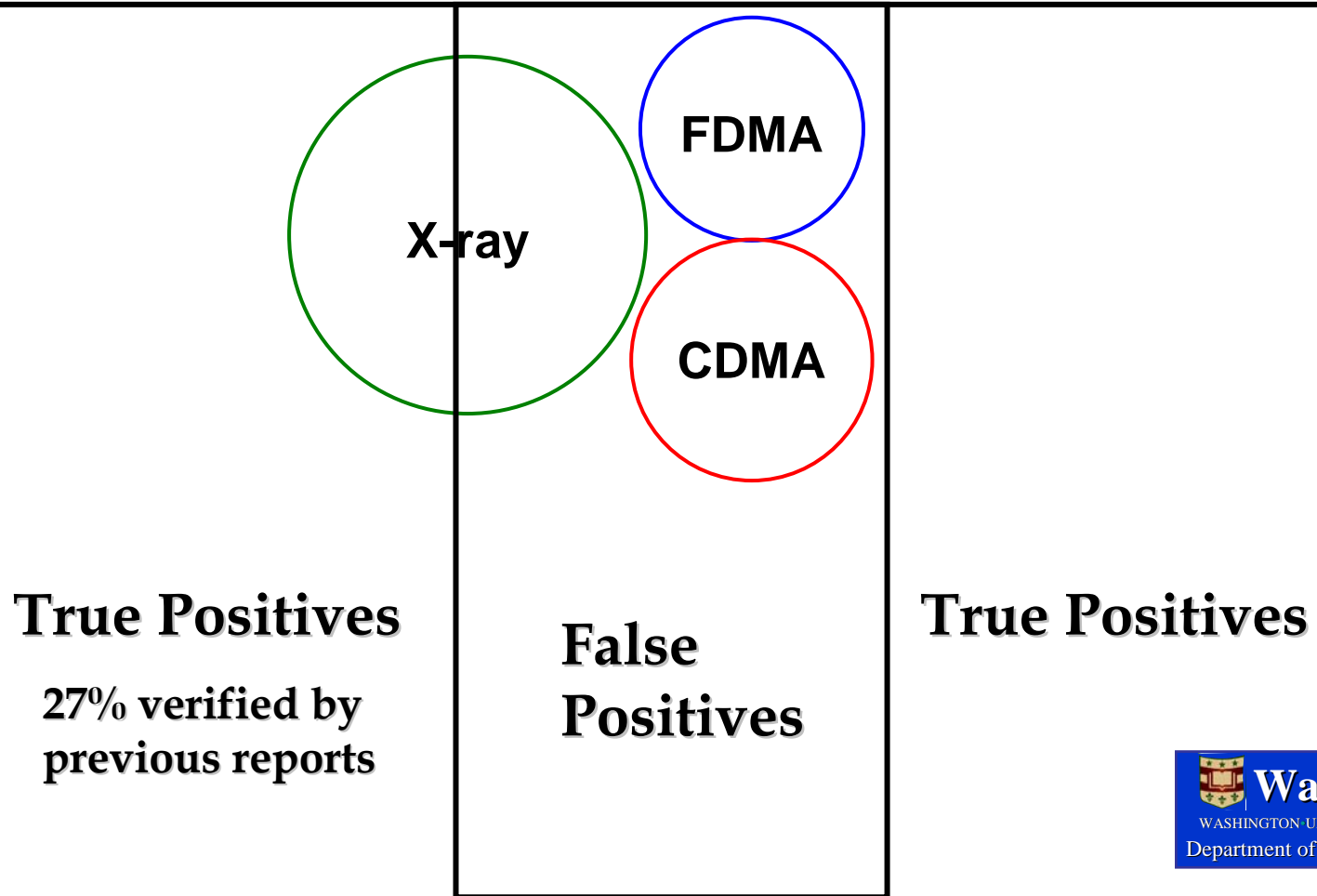
Empirically Measured False Discovery Rates vs. Absolute Fold Change

Absolute Fold Change	Empirically Measured False Discovery Rate		
	CDMA	FDMA	X-ray
>1.0	1.16	1.33	0.51
>1.1	1.16	1.39	0.51
>1.2	1.31	1.48	0.54
>1.3	1.46	1.63	0.60
>1.4	1.38	1.77	0.68
>1.5	1.23	2.06	0.70
>1.6	1.43	2.10	0.76
>1.8	8.20	16.40	0.96

Venn Diagram of 1.3 Fold Up-Regulated Genes



Venn Diagram of 1.3 Fold Down-Regulated Genes



Summary

- 1.** After RF the number of probe sets showing changes in gene expression did not exceed the false positive rate.
- 2.** There were two common genes changing expression in both CDMA and FDMA exposed samples, i.e. expected from chance alone.
- 3.** After 0.68 Gy of X-rays the number of probe sets showing changes in gene expression was ~ twice the false positive rate.
- 4.** After 0.68 Gy, 27-40 % of the number exceeding the false positive rate matched genes reported to be ionizing radiation responsive.

Conclusions

- 1. We did not detect change in gene expression following 24 hr, 5 W/Kg RF exposure above the measured false positive rate.**
- 2. The methods used did detect gene expression changes 4 hr after irradiation with 0.68 Gy of X-rays**

Support: Motorola corp.; US AFOSR-MURI

Transcription and Cell-Cycle Control Genes Up-Regulated after 0.68 Gy Confirmed by Previous Reports

Symbol	Gene Name	Fold ↓	Function/Cellular Component/ Pathway
Dlx5	distal-less homeobox 5	1.4	regulation of transcription, DNA-dependent
Pias1	protein inhibitor of activated STAT	1.4	transcription, regulation of transcription
Ccnb1	cyclin B1, related sequence 1	1.3	regulation of cell cycle
Cdkn1a	cyclin-dependent kinase inhibitor 1A (P21)	2.3	regulation of cell cycle , response to DNA damage stimulus, cell cycle arrest
Ccnd1	cyclin D1	1.3	regulation of cell cycle
Mdm2	transformed mouse 3T3 cell double minute 2	1.5	ubiquitin cycle, traversing start control point of mitotic cell cycle
Cdk4	cyclin-dependent kinase 4	1.4	regulation of cell cycle
Ccng	cyclin G1	1.6	regulation of cell cycle
Wig1	wild-type p53-induced gene 1	1.8	negative regulation of cell growth

Signaling, DNA Repair and Apoptosis Genes Up-Regulated after 0.68 Gy Confirmed by Previous Reports

Symbol	Gene Name	Fold ↓	Function/Cellular Component/ Pathway
Gja1	gap junction membrane channel protein alpha 1	2.3	cell communication, cell-cell signaling
Rasa3	RAS p21 protein activator 3	1.4	intracellular signaling cascade
Ercc5	excision repair cross-complementing rodent repair deficiency	1.6	DNA repair, nucleotide-excision repair, response to DNA damage stimulus
Rad1	RAD1 homolog (S. pombe)	1.4	DNA repair
Bax	Bcl2-associated X protein	1.6	DNA damage induced apoptosis
Tnfrsf6	Tumor necrosis factor super family member 6 (Fas)	1.6	apoptosis, induction of apoptosis via death domain receptors

Metabolism, Transport and Other Genes Up-Regulated after 0.68 Gy Confirmed by Previous Reports

Symbol	Gene Name	Fold ↓	Function/Cellular Component/ Pathway
Fuca1	fucosidase, alpha-L- 1, tissue	1.3	carbohydrate metabolism
Mgst3	microsomal glutathione S-transferase 3	1.3	transferase activity
Txn12	thioredoxin-like 2	1.4	electron transport
Ggh	gamma-glutamyl hydrolase	1.3	redox processing
Nono	non-POU-domain-containing, octamer binding protein	1.3	nucleic acid binding, DNA binding, RNA binding
Arf3	ADP-ribosylation factor 3	1.8	protein transport
Dnajc8	DnaJ (Hsp40) homolog, subfamily C, member 8	1.3	protein folding
Mrpl54	mitochondrial ribosomal protein L5	1.3	mitochondrial protein synthesis
Vcl	vinculin	1.4	cell adhesion, regulation of cell migration

Transcription Related Genes Down-Regulated after 0.68 Gy

Confirmed by Previous Reports

Symbol	Gene Name	Fold ↓	Function/Cellular Component/ Pathway
Gtf3a	general transcription factor III A	1.3	Transcription factor
Mtf2	metal response element binding transcription factor 2	1.3	Transcription factor
Ncoa3	nuclear receptor coactivator 3	1.4	Regulate post IR transcription
Synj2bp	synaptojanin 2 binding protein	1.4	RNA binding
Taf9	TAF9 RNA polymerase II, TATA box binding protein-associated factor	1.4	Regulation of transcription
Asfla	ASF1 anti-silencing function 1 homolog A (<i>S. cerevisiae</i>)	1.9	DNA repair, nucleosome assembly, coordination of transcription and S-phase transit, down regulated due to S-phase check point, reduced level keeps chromatin open

Signaling and Cell-Cycle Control Genes Down-Regulated after 0.68 Gy Confirmed by Previous Reports

Symbol	Gene Name	Fold ↓	Function/Cellular Component/ Pathway
Cdc6	cell division cycle 6 homolog (S. cerevisiae)	1.6	DNA replication/mitosis, down-regulation of proliferation
Chek1	checkpoint kinase 1 homolog (S. pombe)	1.5	DNA damage checkpoint G2/M
Dab2	disabled homolog 2 (Drosophila)	1.3	Cell proliferation
Dusp19	dual specificity phosphatase 19	1.3	Signal transduction
Sh3glb1	SH3-domain GRB2-like B1 (endophilin)	1.4	Synaptic signaling
Usp9x	ubiquitin specific protease 9, X chromosome	1.4	Development