High Throughput Calcium Screening in Human Neutrophils; A Possibility in 1536 Well Format using FLIPR^{TETRA}

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What did we do?

- Isolated Human neutrophils from “normal” donors
- In 1536 well format we examined:
  - Pharmacology of the agonist (versus 384)
  - Pharmacology of 14 standard antagonists (versus historical 384 data)
  - Correlated 5,500 compounds (triplicate) against EC80 (retest screening)

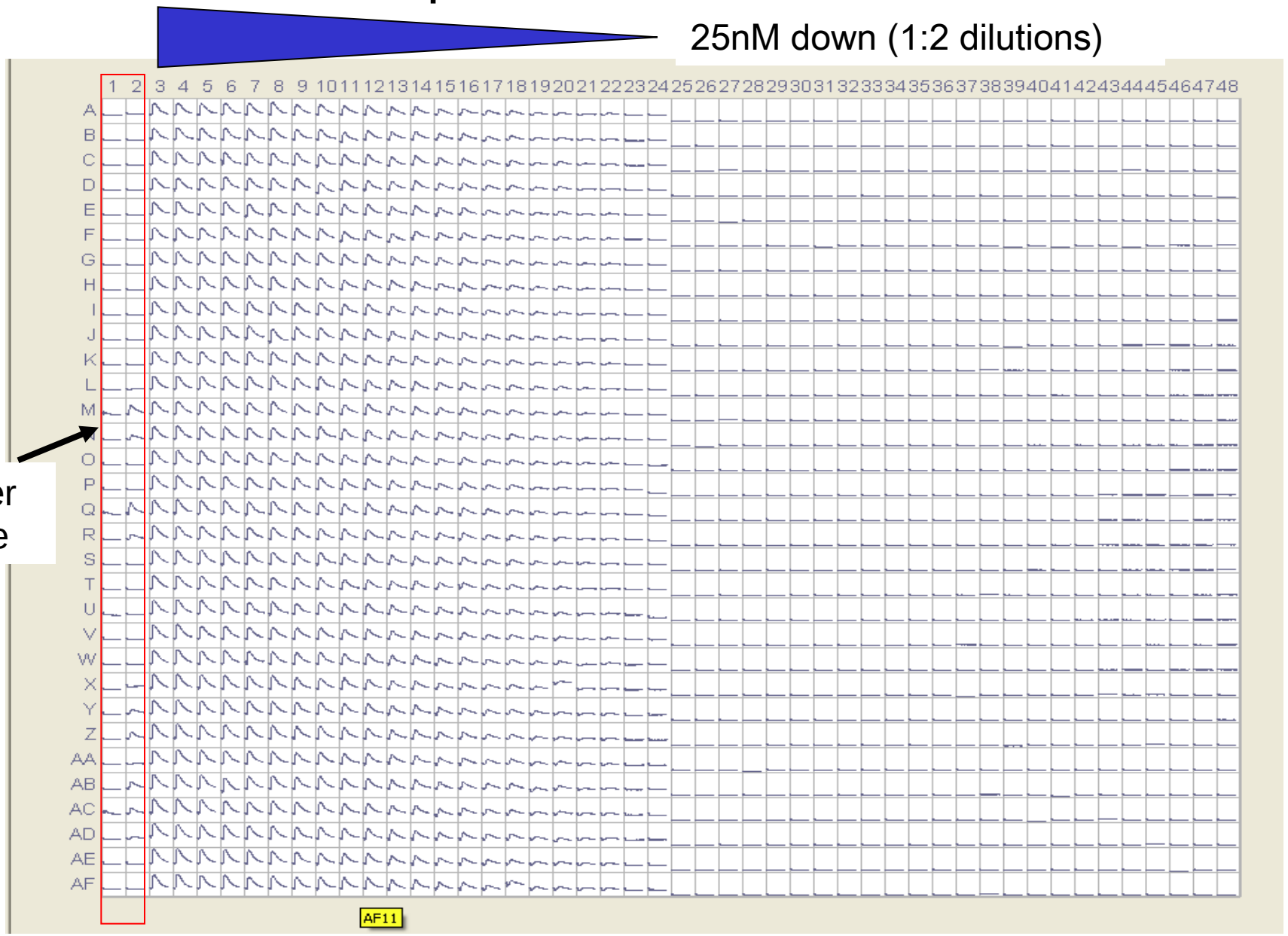
How did we do it? - Equipment

- Tetra with 1536 well head, used 1 gasket per day to add agonist from 1536 well plate to cell plate
- Dispensed cells using DW4
- Dispensed compounds using Echo 550 (Labcyte)
- Correlated data using Spotfire and EC50’s etc using Origin software

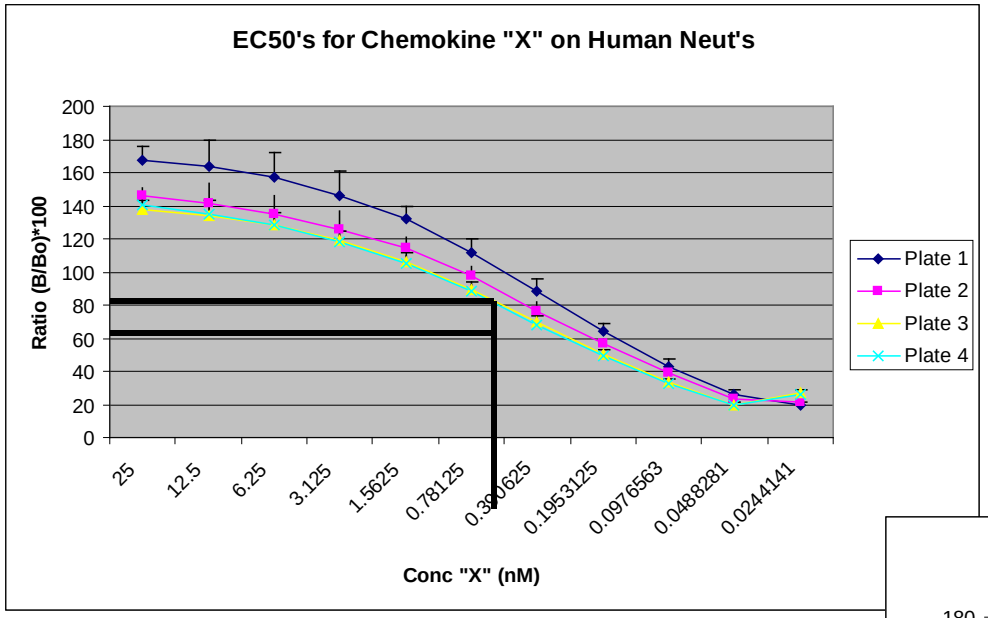
Experimental Protocol

- Isolate human neutrophils using density gradient centrifugation (*) into Tyrodes as final assay buffer @ 4 million/ml
- Add Fluo-4 (2uM final) and MDC red quencher (1:1000)
- Dispense 5ul/well (20K/well)
- Centrifuge in micro centrifuge for 20 secs
- Incubate on bench for between 30 and 60 mins
- Compound additions (50nl – 100% DMSO) were either performed before or after cell addition
- Read in Tetra with addition of 2ul agonist, ATP (10uM) or control to wells

Screenshot from Tetra; Human neutrophils - Dose response to Chemokine “X”

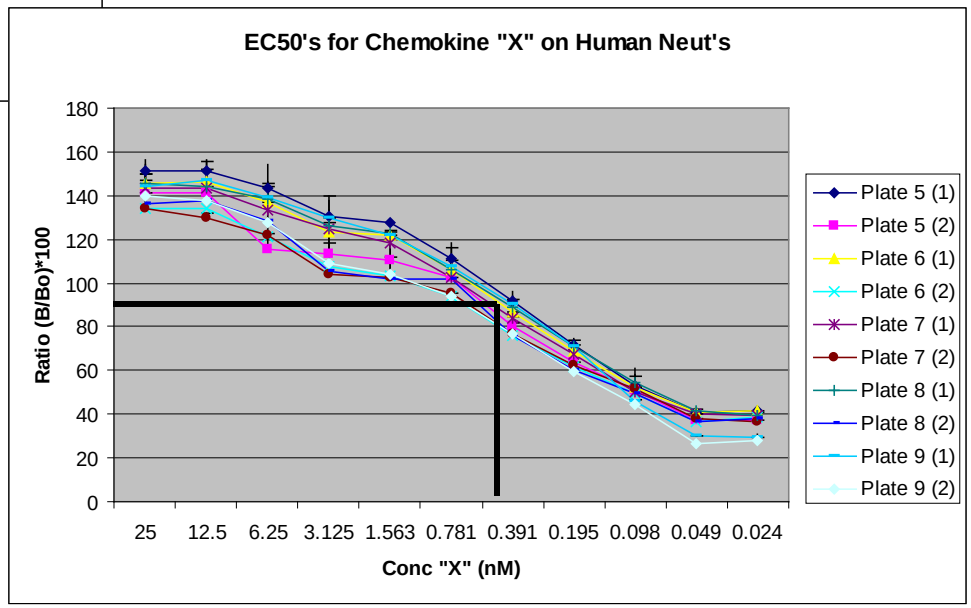


pIC50 of Chemokine “X” on human neutrophils in 1536; Day 1

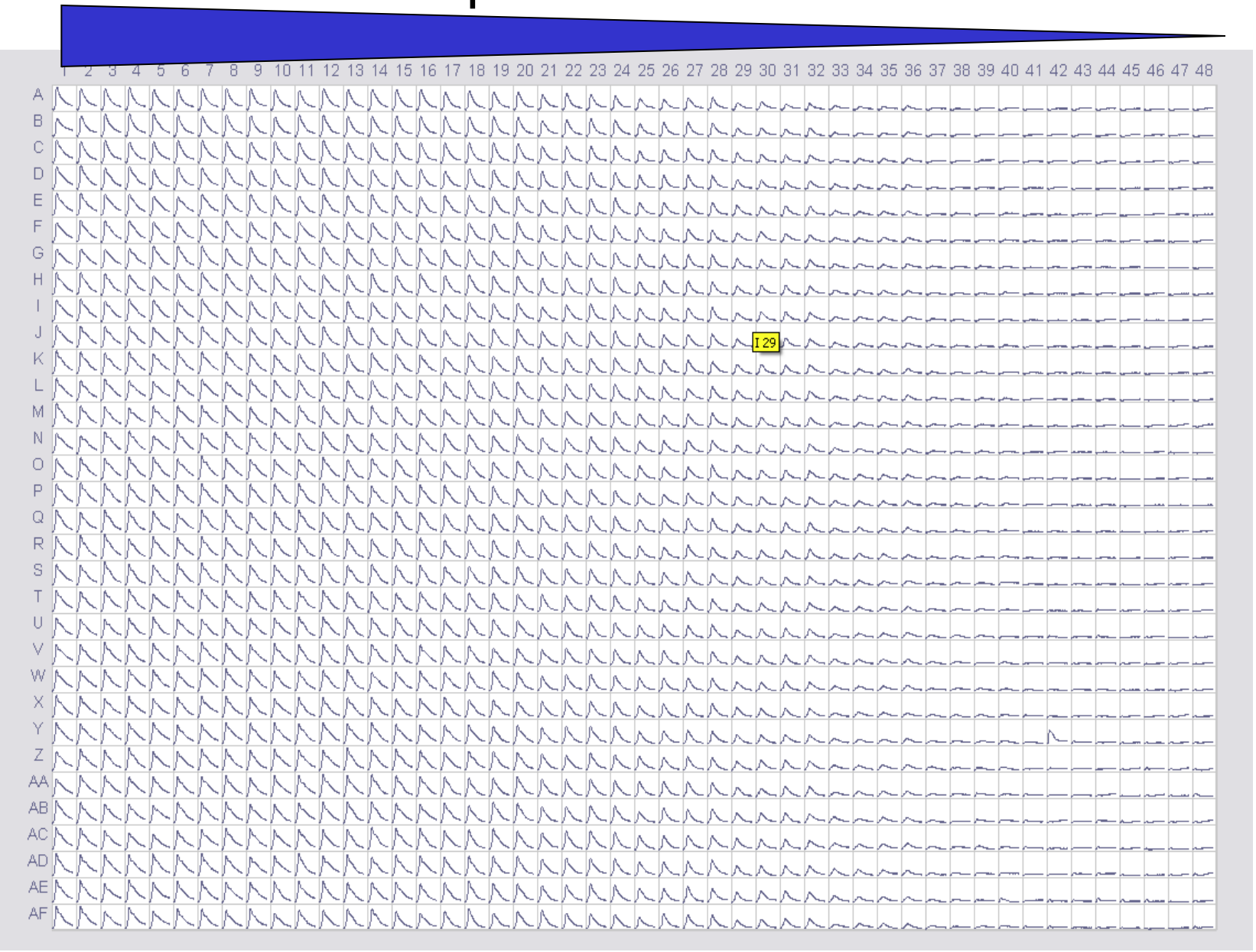


EC50 = 0.4 nM
(In cell line = 0.3 to 0.5 nM

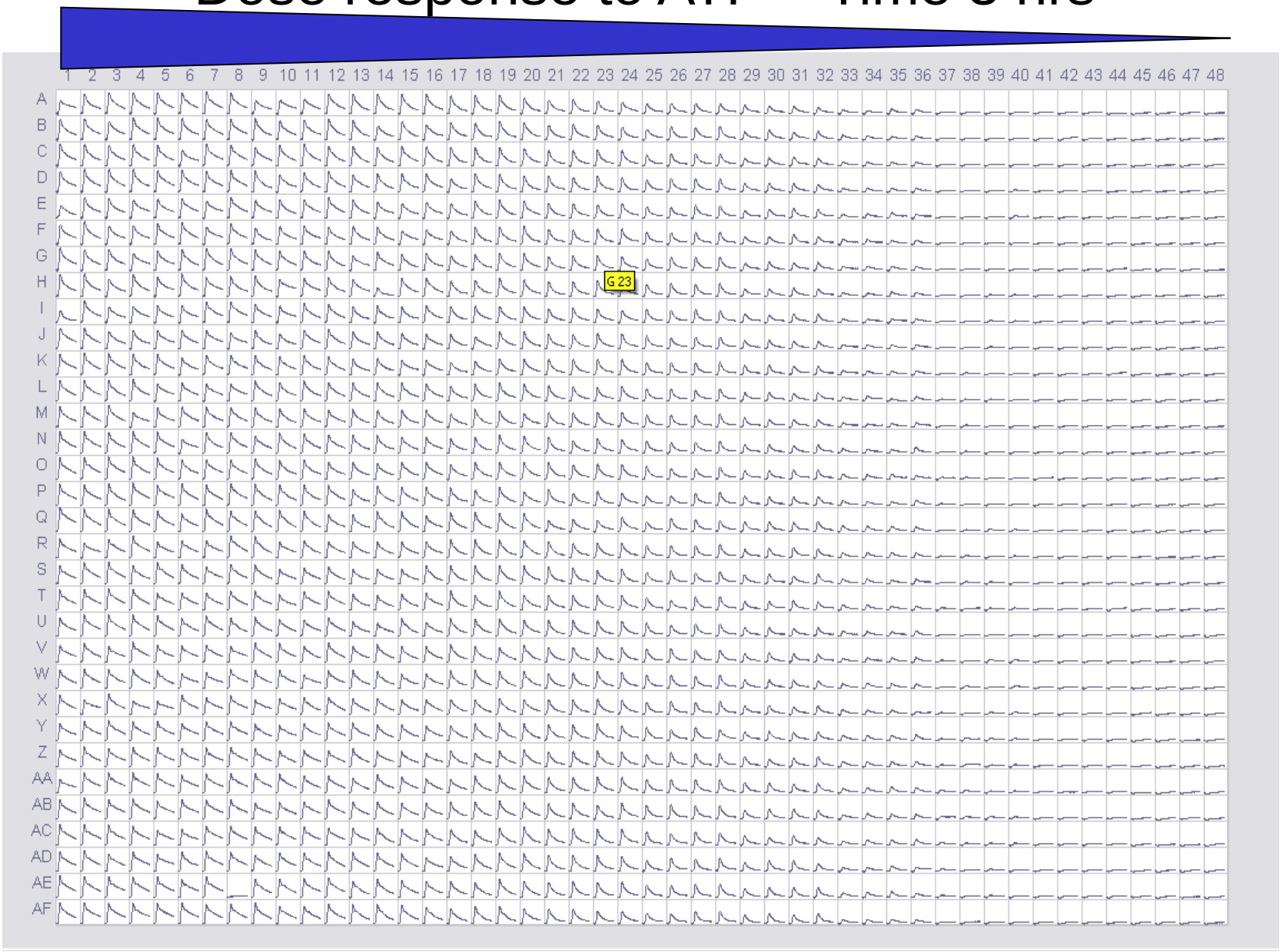
Data not shown for Days 2 and 3 but were very similar



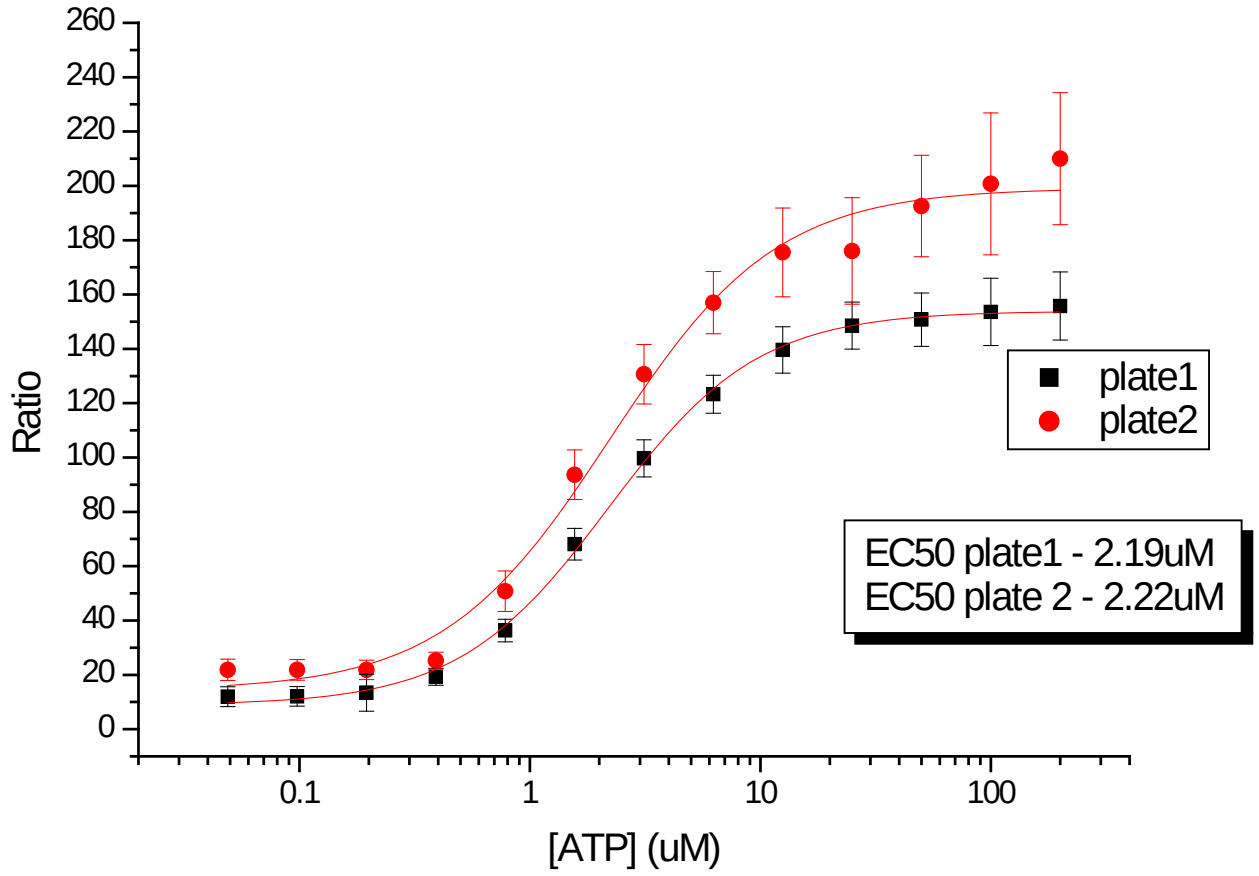
Screenshot from Tetra; Human Neutrophils - Dose response to ATP – Time 0



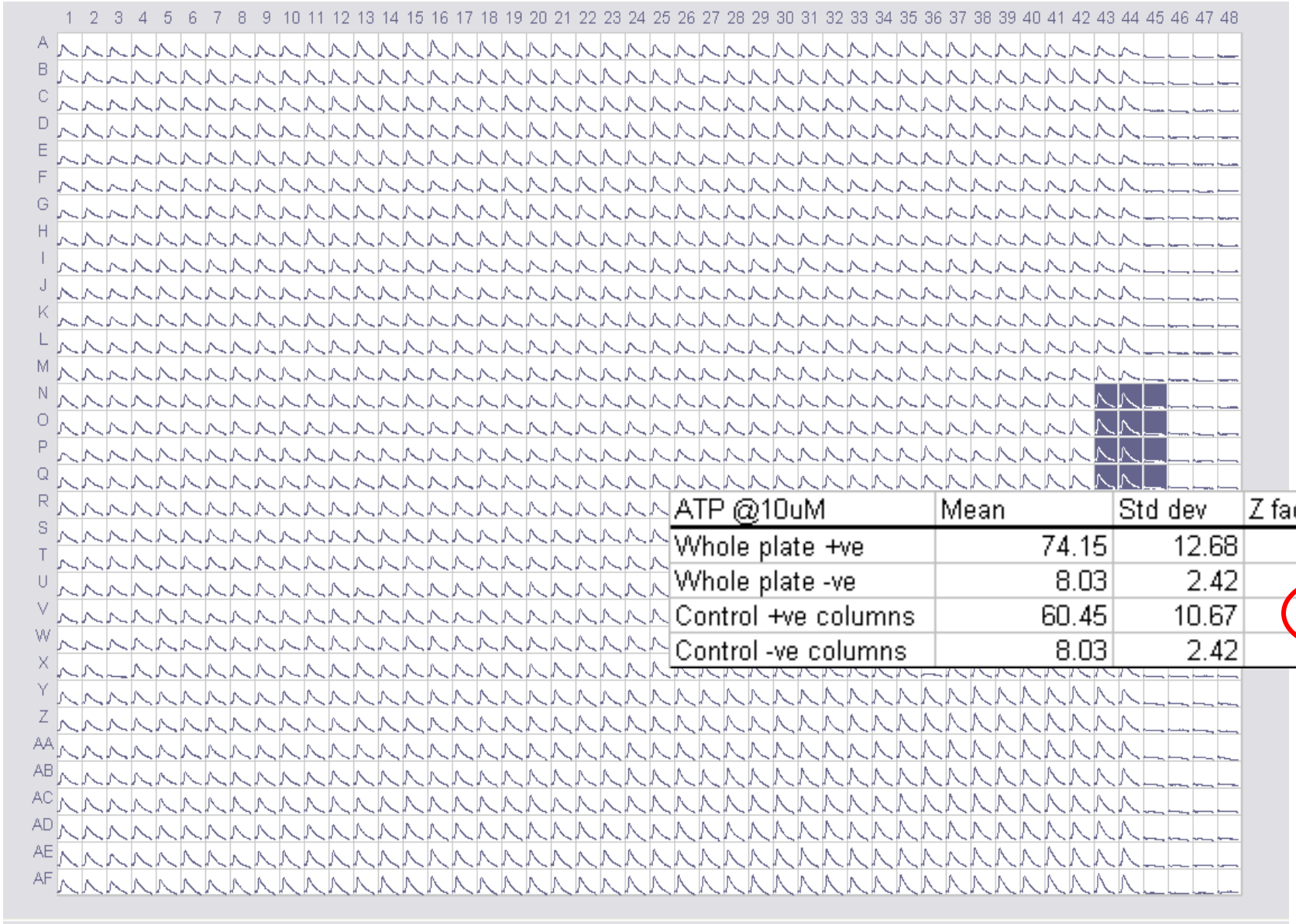
Screenshot from Tetra; Human Neutrophils - Dose response to ATP – Time 3 hrs



EC50 curves for ATP on Human Neutrophils

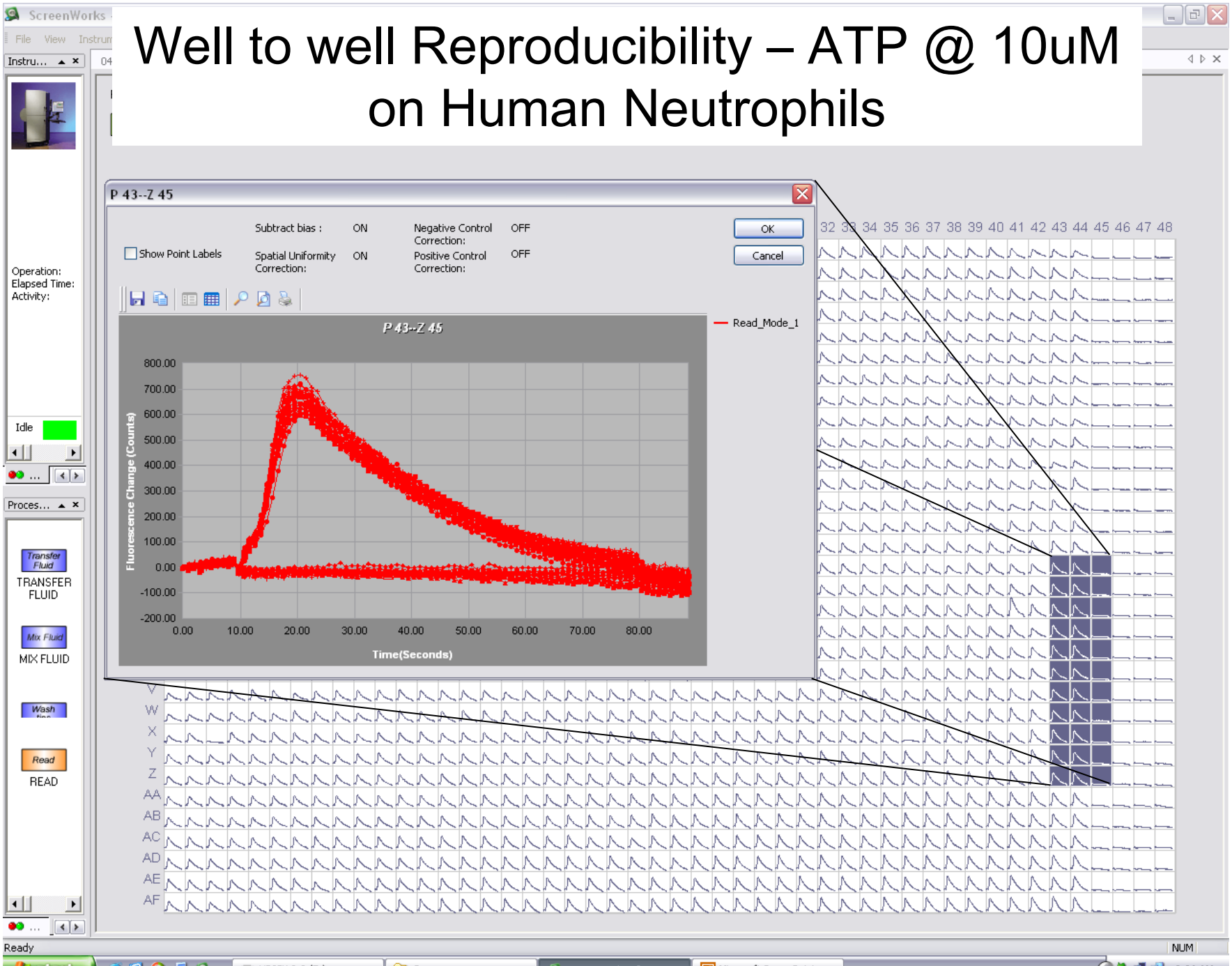


ATP response in Human Neutrophils (@10uM) (1 thru 44) plus controls (45 thru 48)

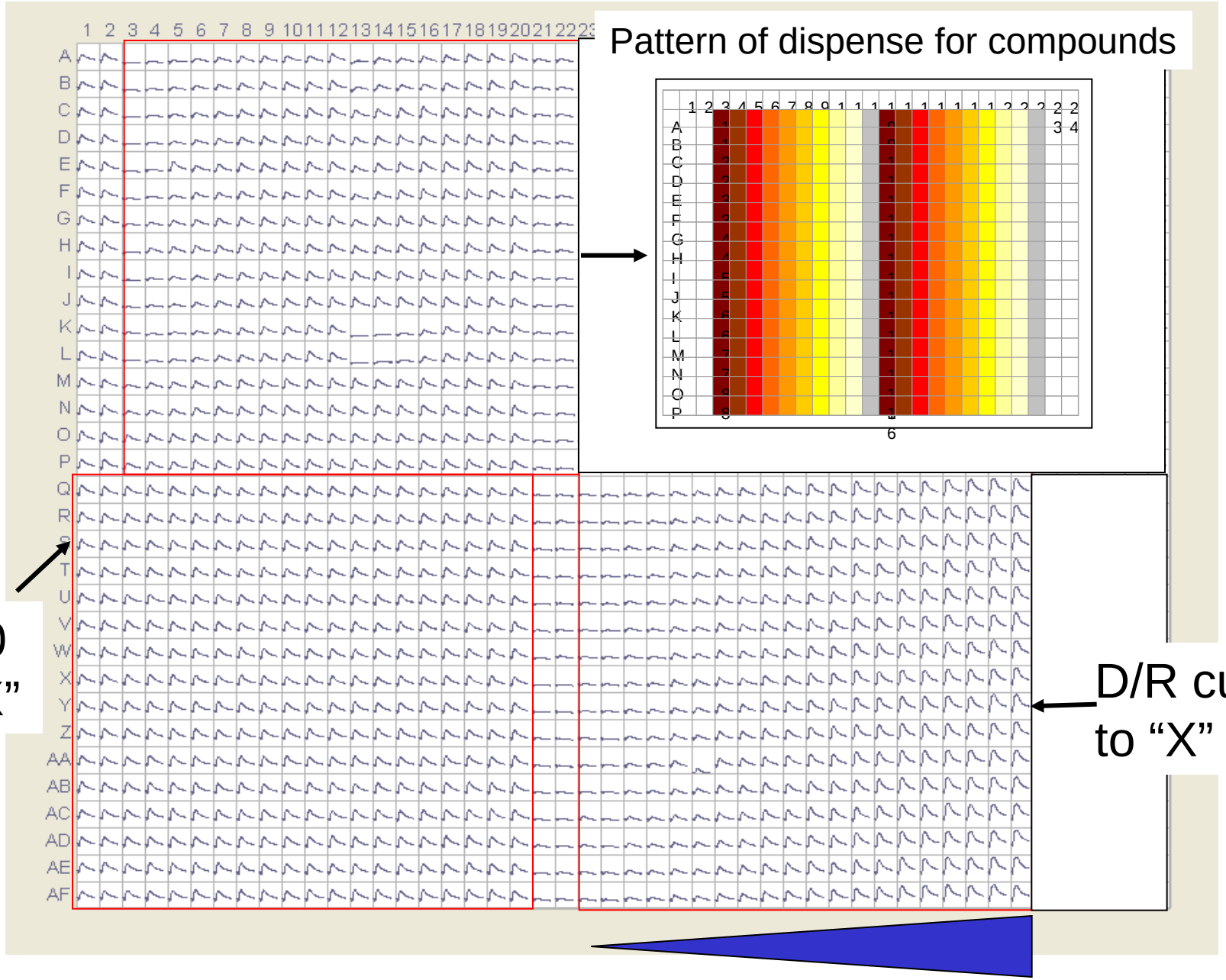


ATP @10uM	Mean	Std dev	Z factor
Whole plate +ve	74.15	12.68	0.32
Whole plate -ve	8.03	2.42	
Control +ve columns	60.45	10.67	0.53
Control -ve columns	8.03	2.42	

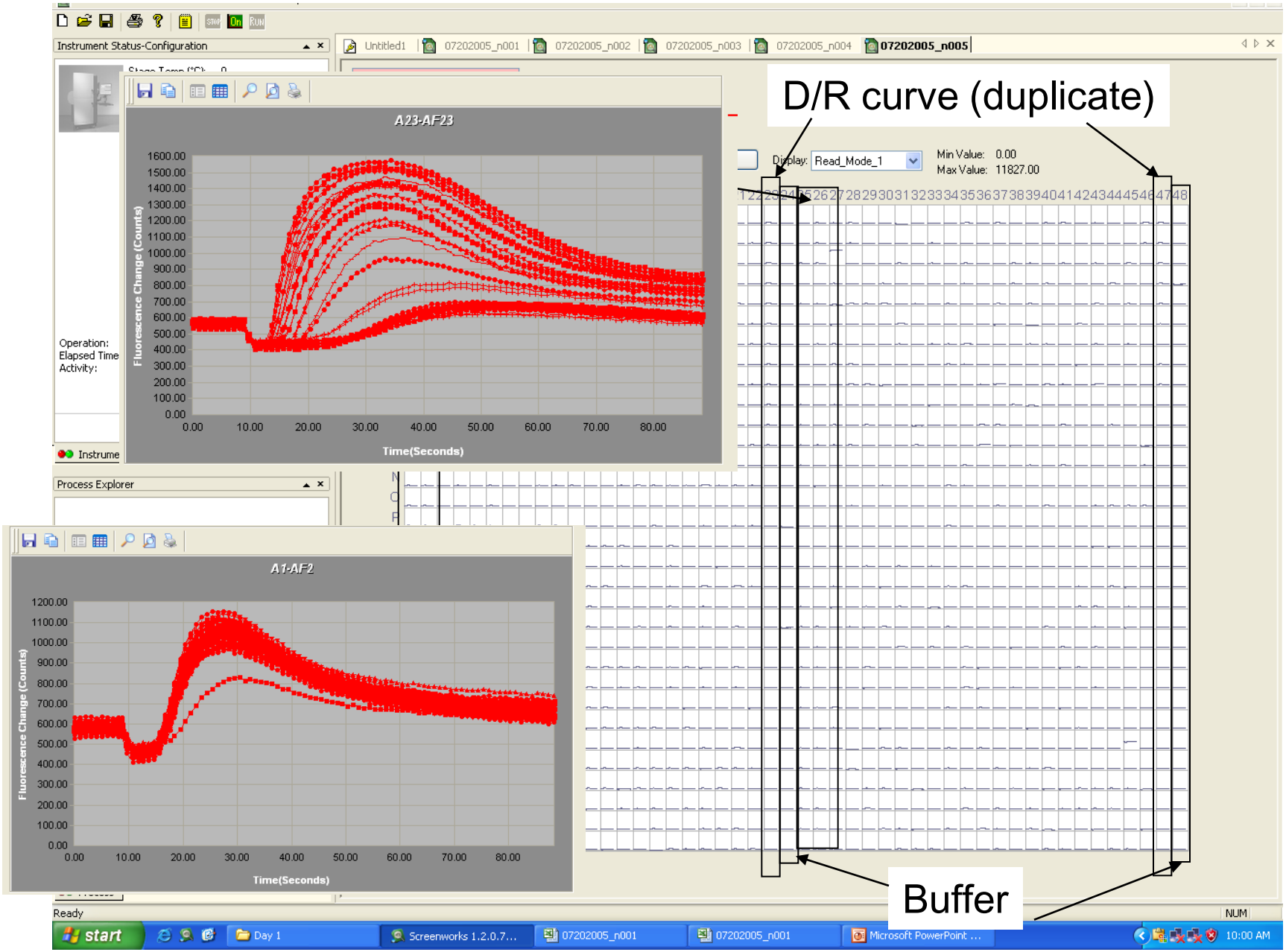
Well to well Reproducibility – ATP @ 10uM on Human Neutrophils



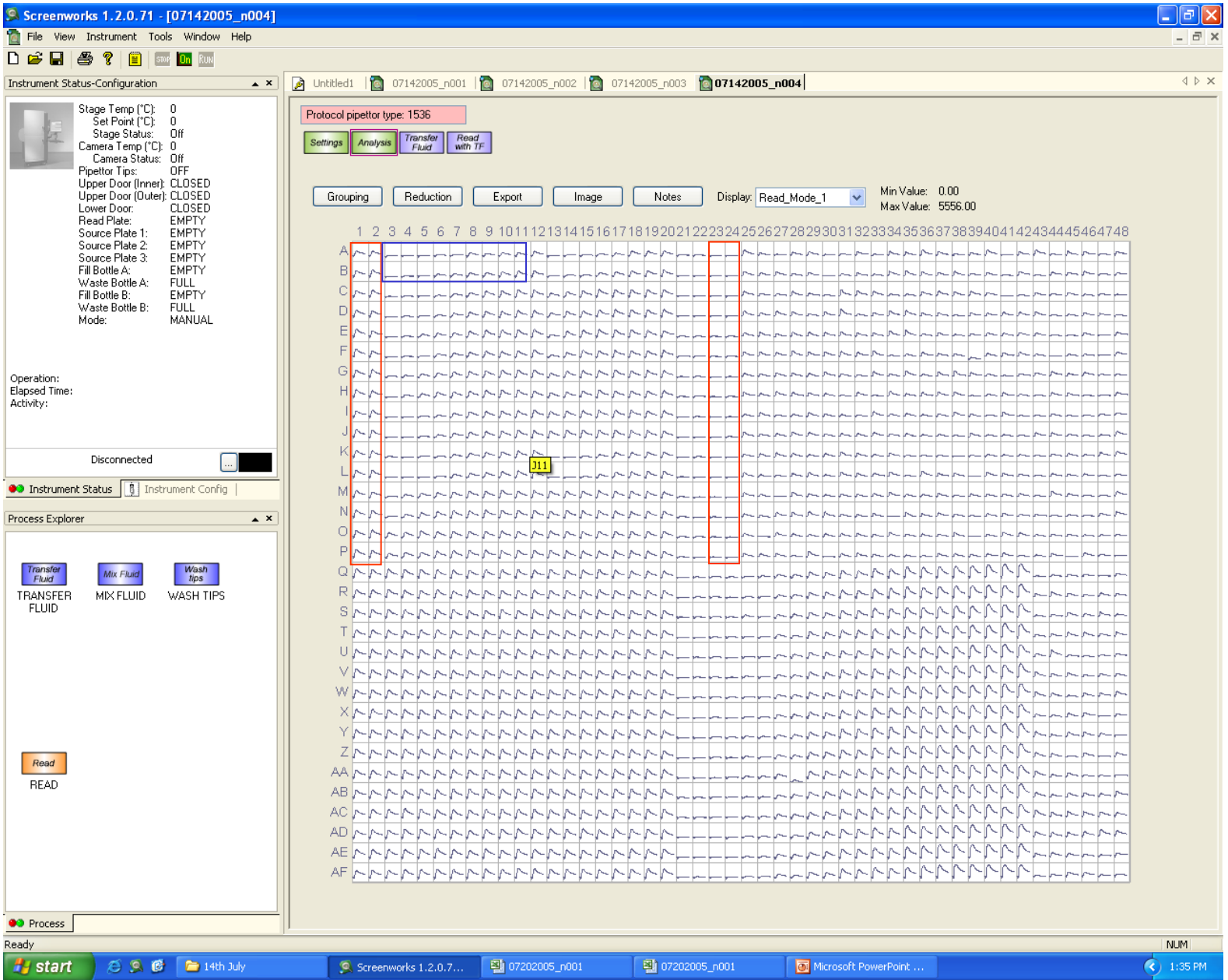
Echo generated compound plate layout



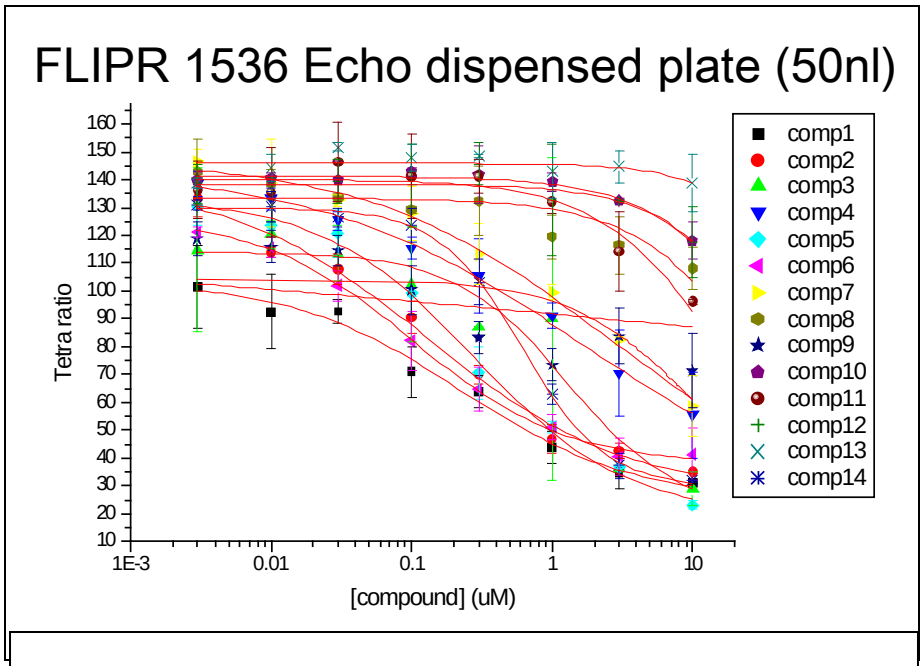
Screenshot from Tetra; screening – day 1

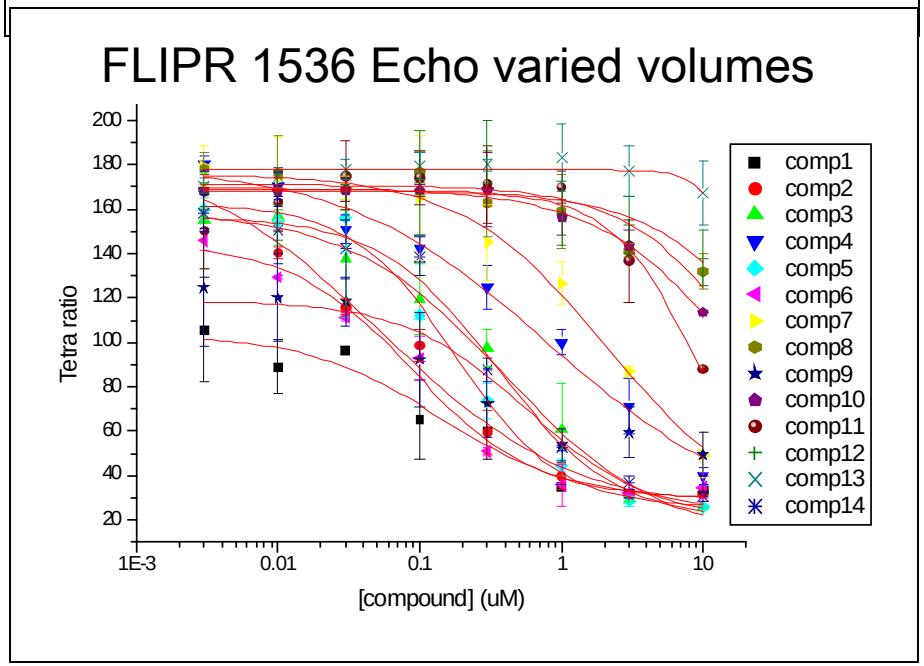


Echo generated compound plate



1536 50nl versus varied volumes

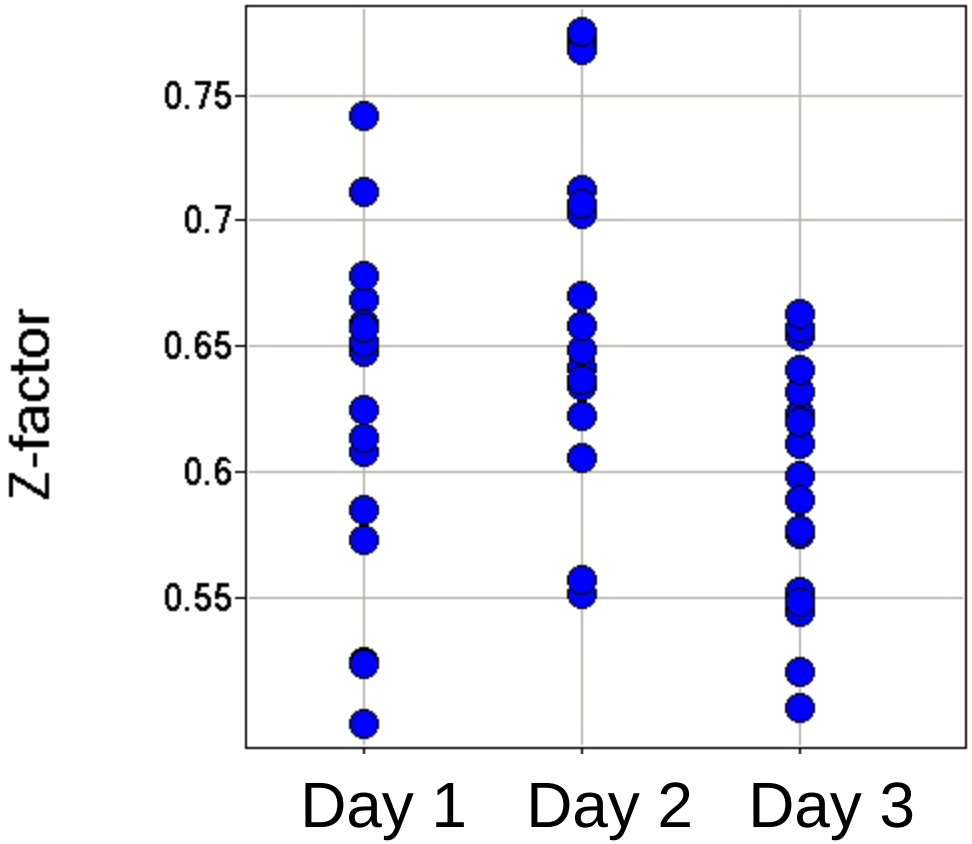




Compound	pIC50 400nl	pIC50 Various
1	6.9	6.7
2	7.3	6.9
3	6.4	5.9
4	6.1	5.8
5	6.7	6.6
6	7.0	7.0
7	5.7	5.6
8	4.6	4.5
9	6.2	5.0
10	4.7	4.4
11	5.1	4.8
12	4.4	4.4
13	4.7	4.0
14	6.5	6.2

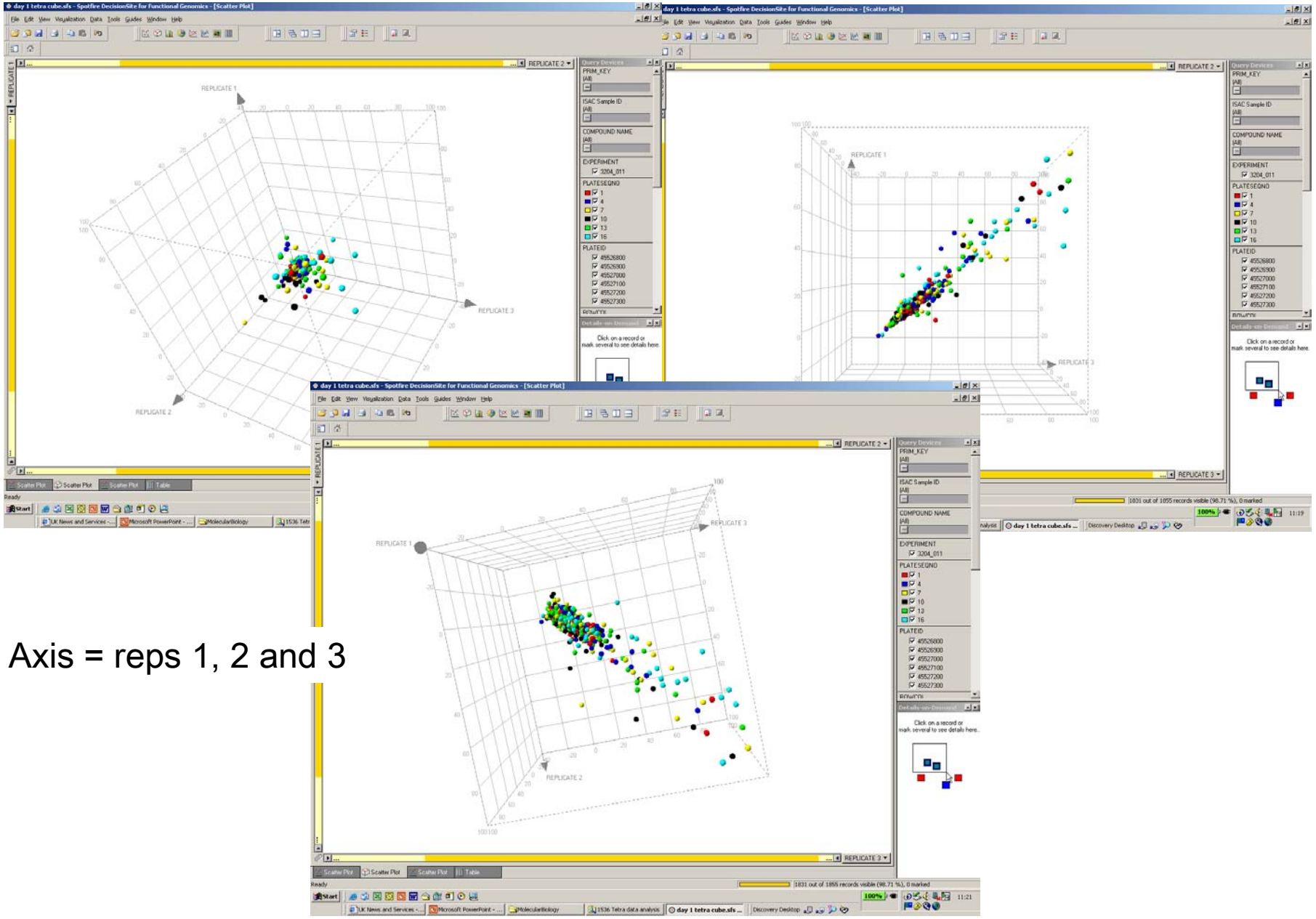
Z Factors each day on each deconvoluted 1536 well plate

384 – n=32 control +ve and -ve



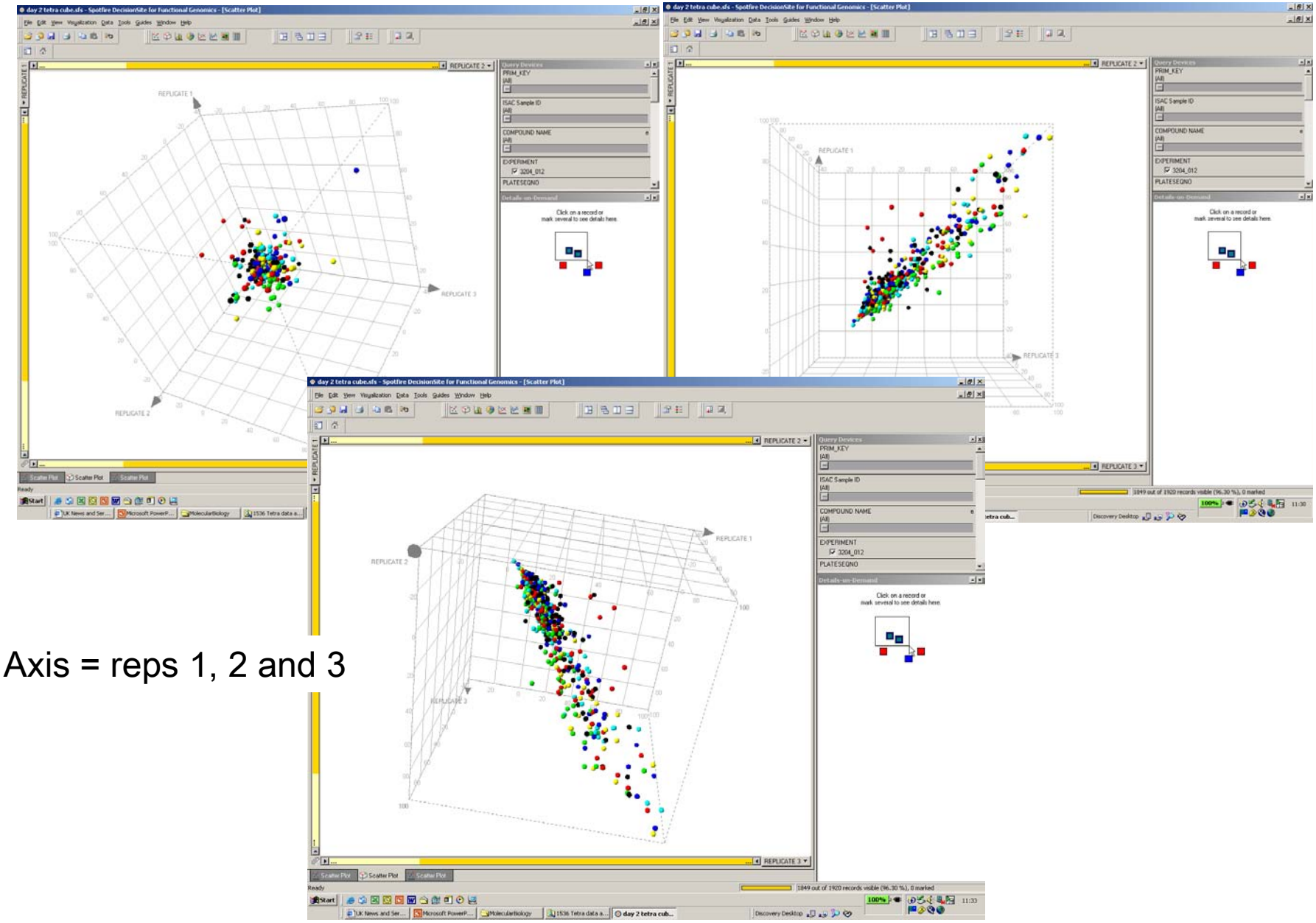
1536 – n=128 control +ve and -ve

Tetra human neutrophils screening day 1 triplicates



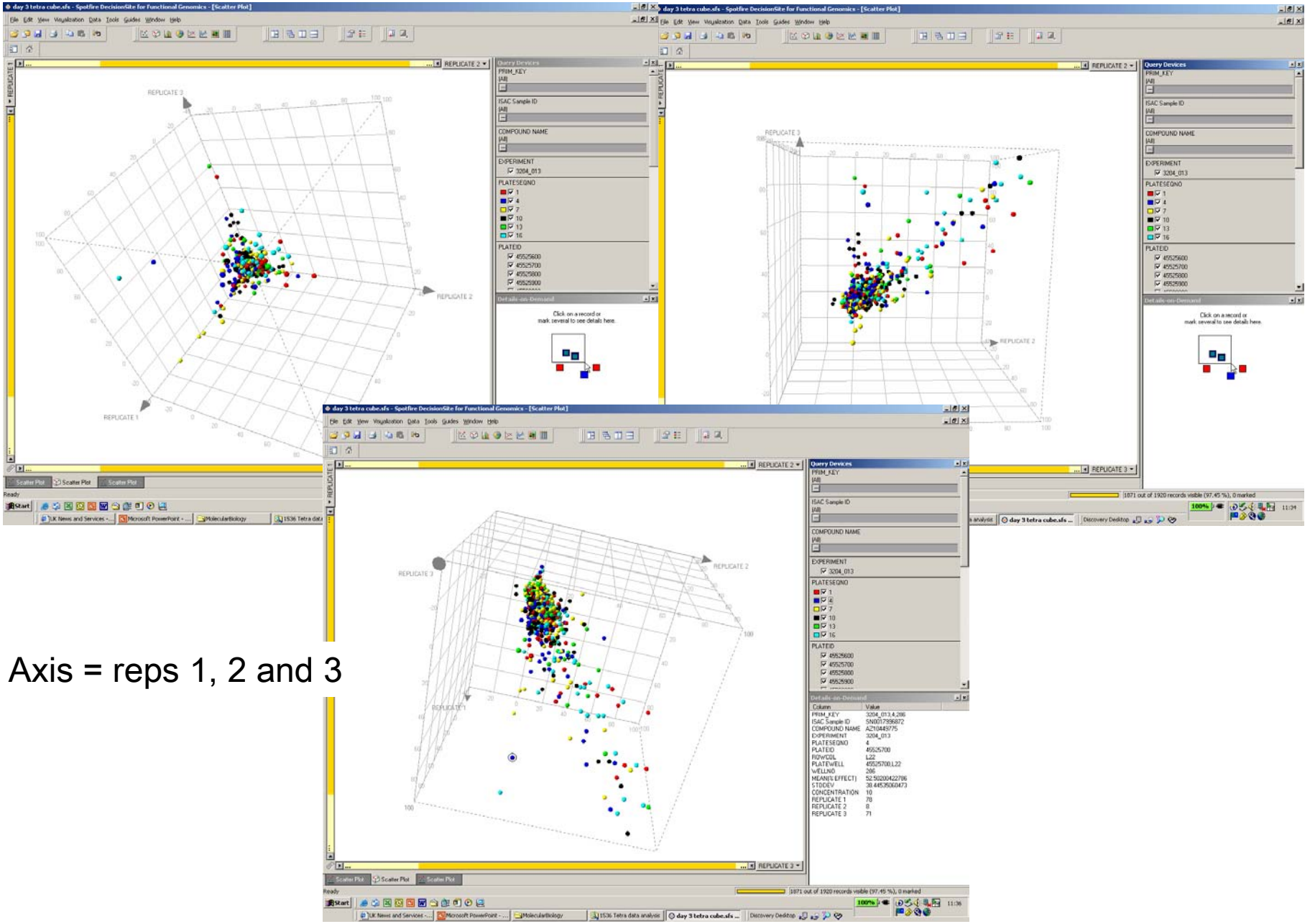
Axis = reps 1, 2 and 3

Tetra human neutrophils screening day 2 triplicates



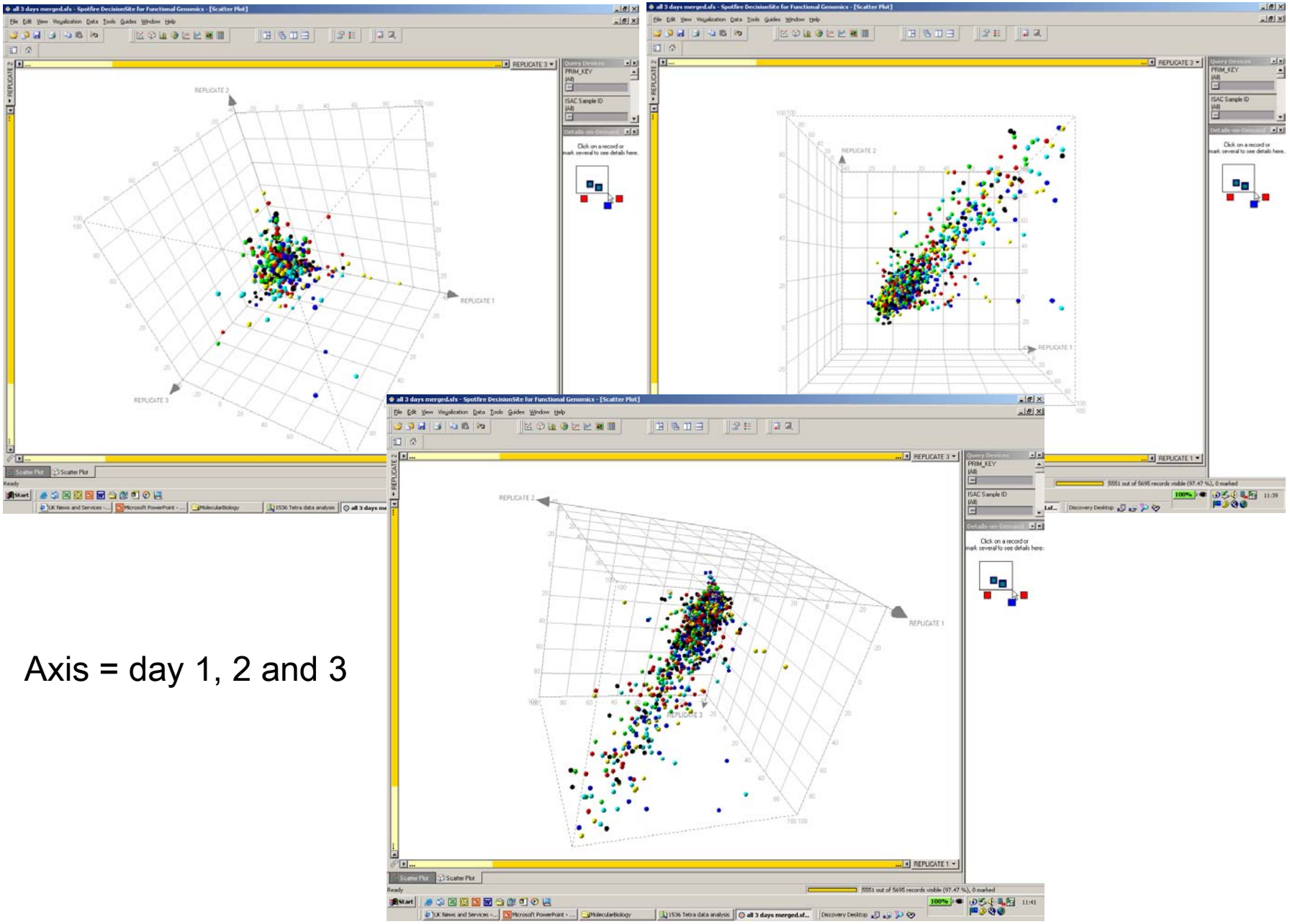
Axis = reps 1, 2 and 3

Tetra human neutrophils screening day 3 triplicates



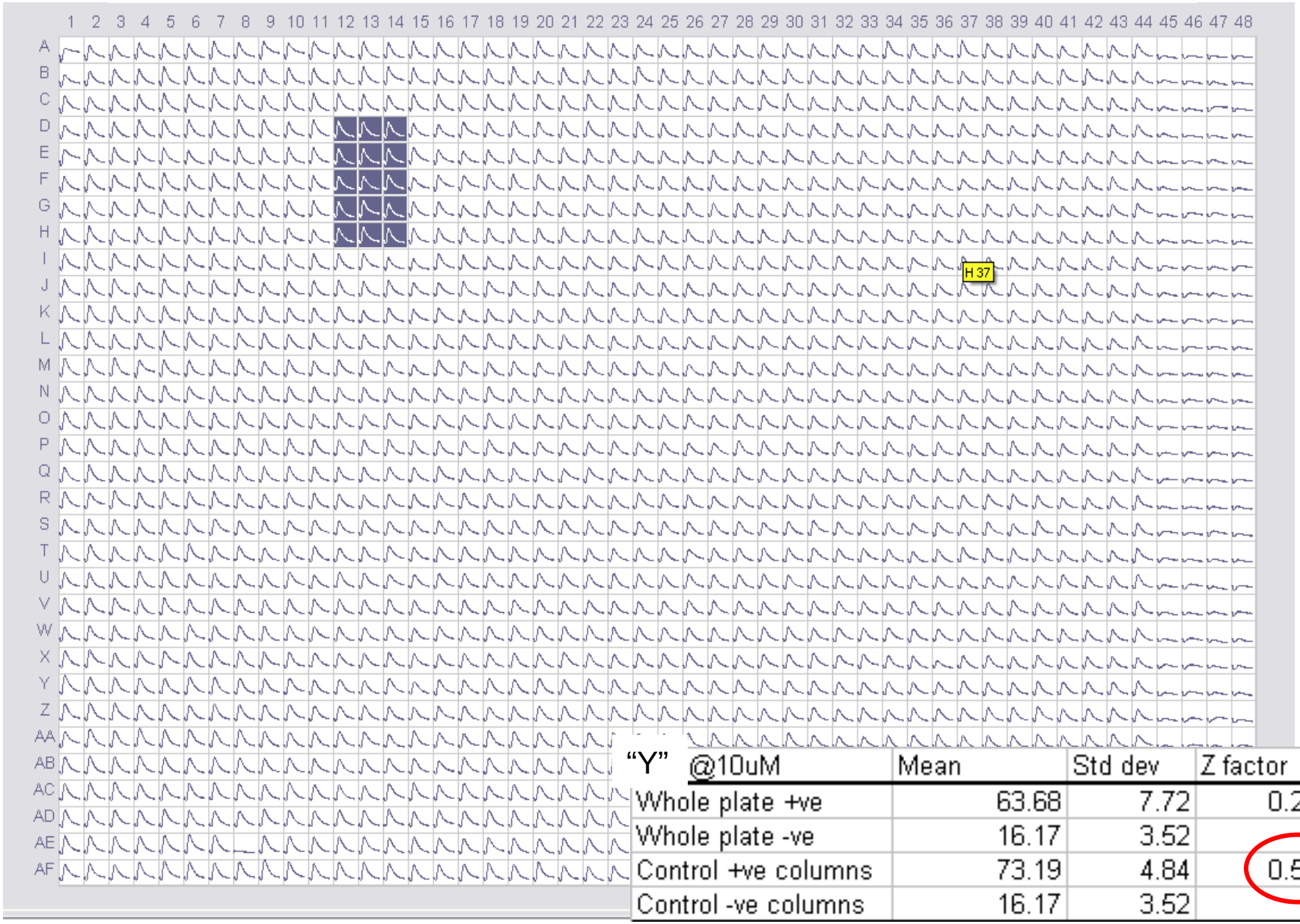
Axis = reps 1, 2 and 3

Tetra human neutrophils screening all 3 days



Axis = day 1, 2 and 3

Second Example: Chemokine “Y” response in Human Neutrophils (EC80) 1 thru 44 plus controls



“Y” @10uM	Mean	Std dev	Z factor
Whole plate +ve	63.68	7.72	0.29
Whole plate -ve	16.17	3.52	
Control +ve columns	73.19	4.84	0.56
Control -ve columns	16.17	3.52	

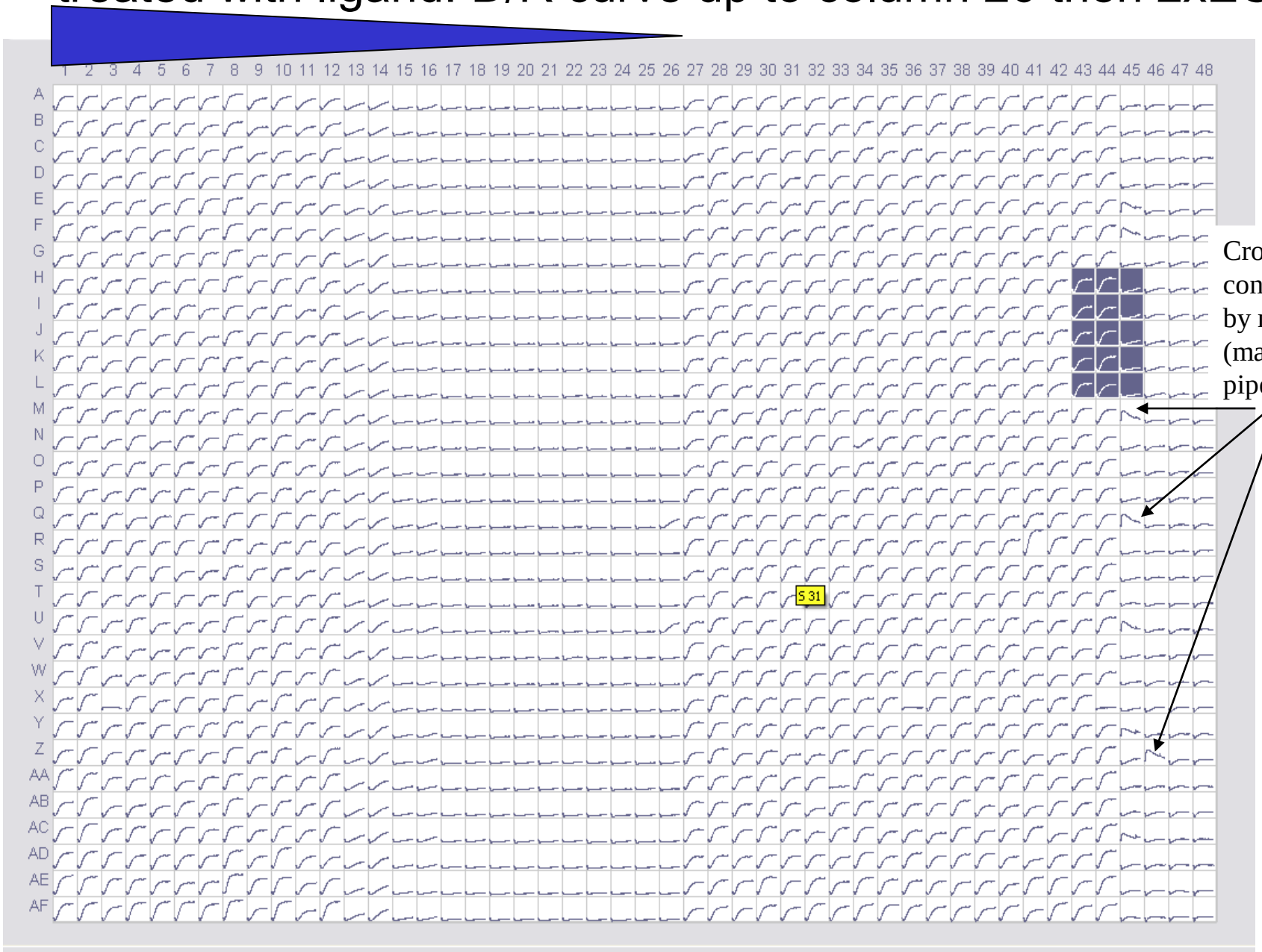
What about cell line work?

HEK 293 cells and CHO cells

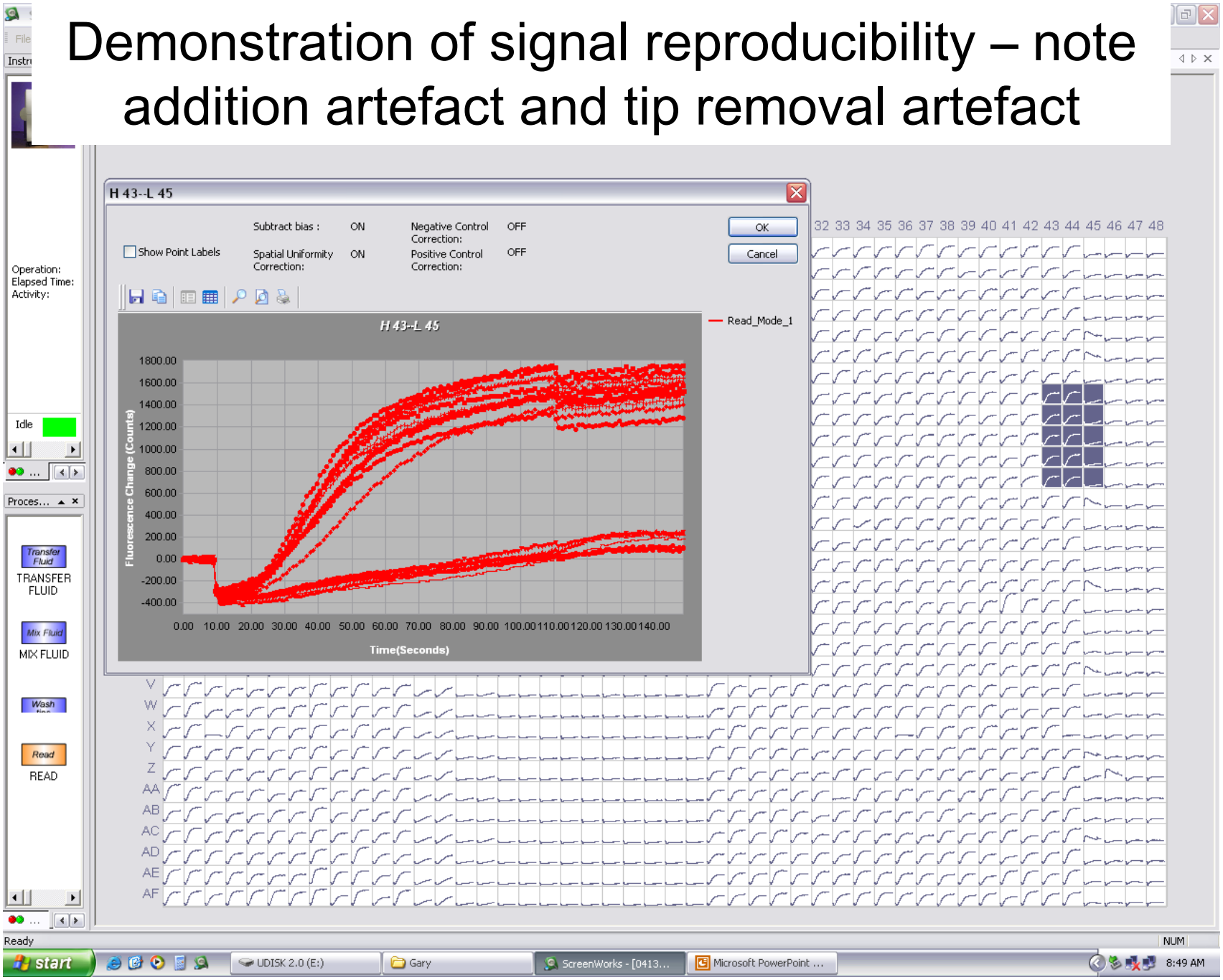
Method

- Isolated CHO cells, diluted to 4.0 million /ml and plated out into Greiner 1536 well low base plates using DW4 dispenser
- Incubate for 24 hrs
- Aspirate media (DW4), dispense 5ul loading buffer containing Fluo-4 and quencher (normal conditions) – discuss equipment used to perform this task.
- Incubated for 45 mins @ 37^{o}C
- Read and dispensed 2ul of agonist to wells on Tetra

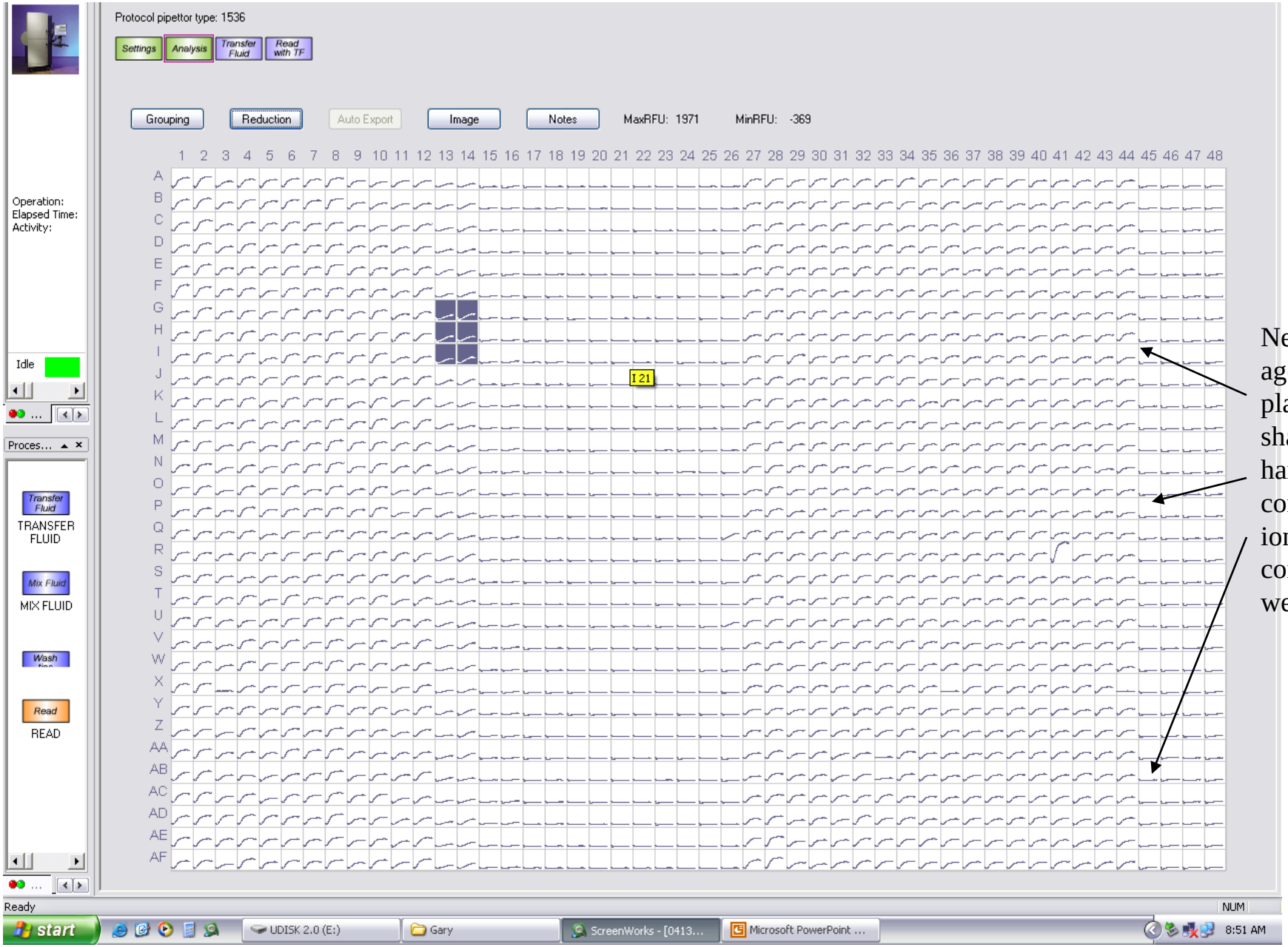
CHO cells (2K/well) stably expressing receptor “A” treated with ligand: D/R curve up to column 26 then 2xEC50



Demonstration of signal reproducibility – note addition artefact and tip removal artefact

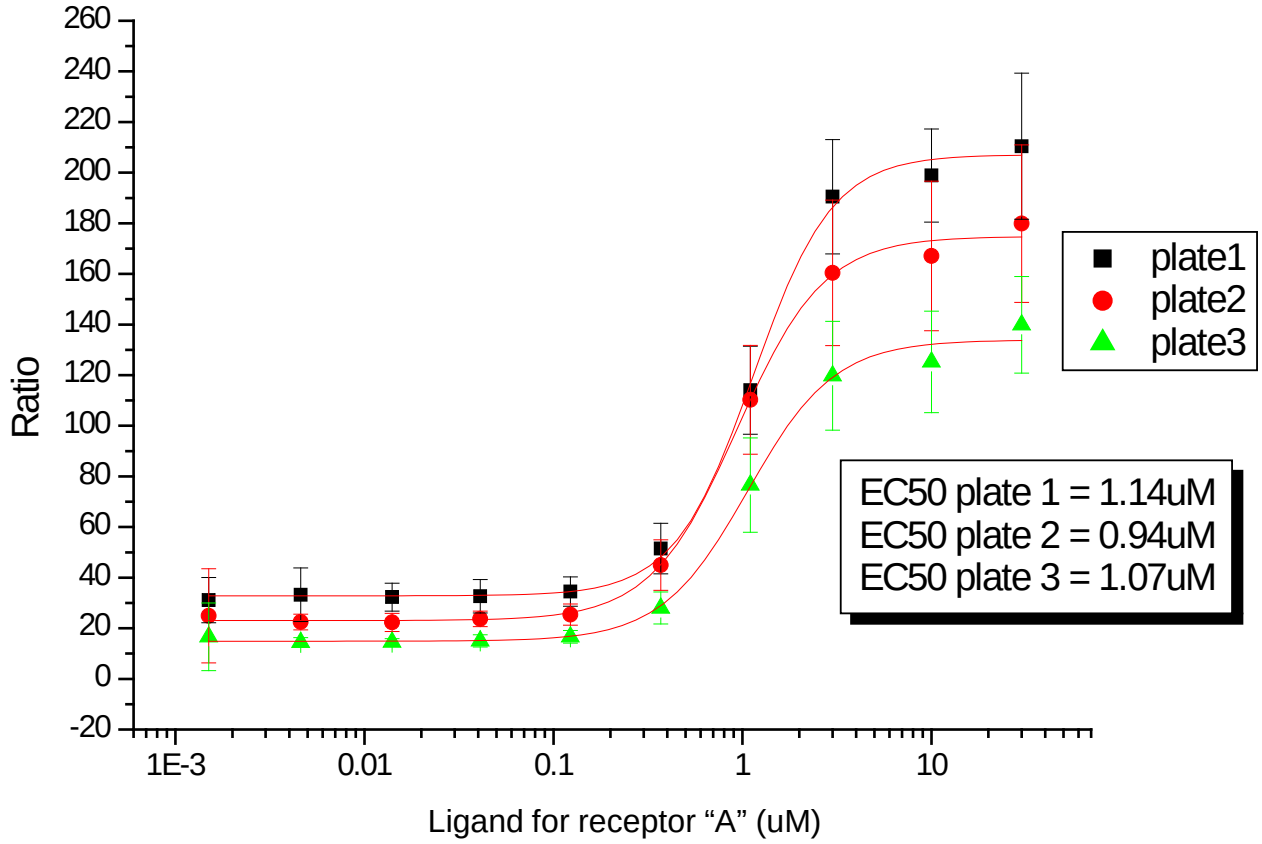


New agonist plate – note no responses in control wells



New agonist plate – less shakey hands = no contamination in control wells!

Receptor “A” (CHO cells) in 1536



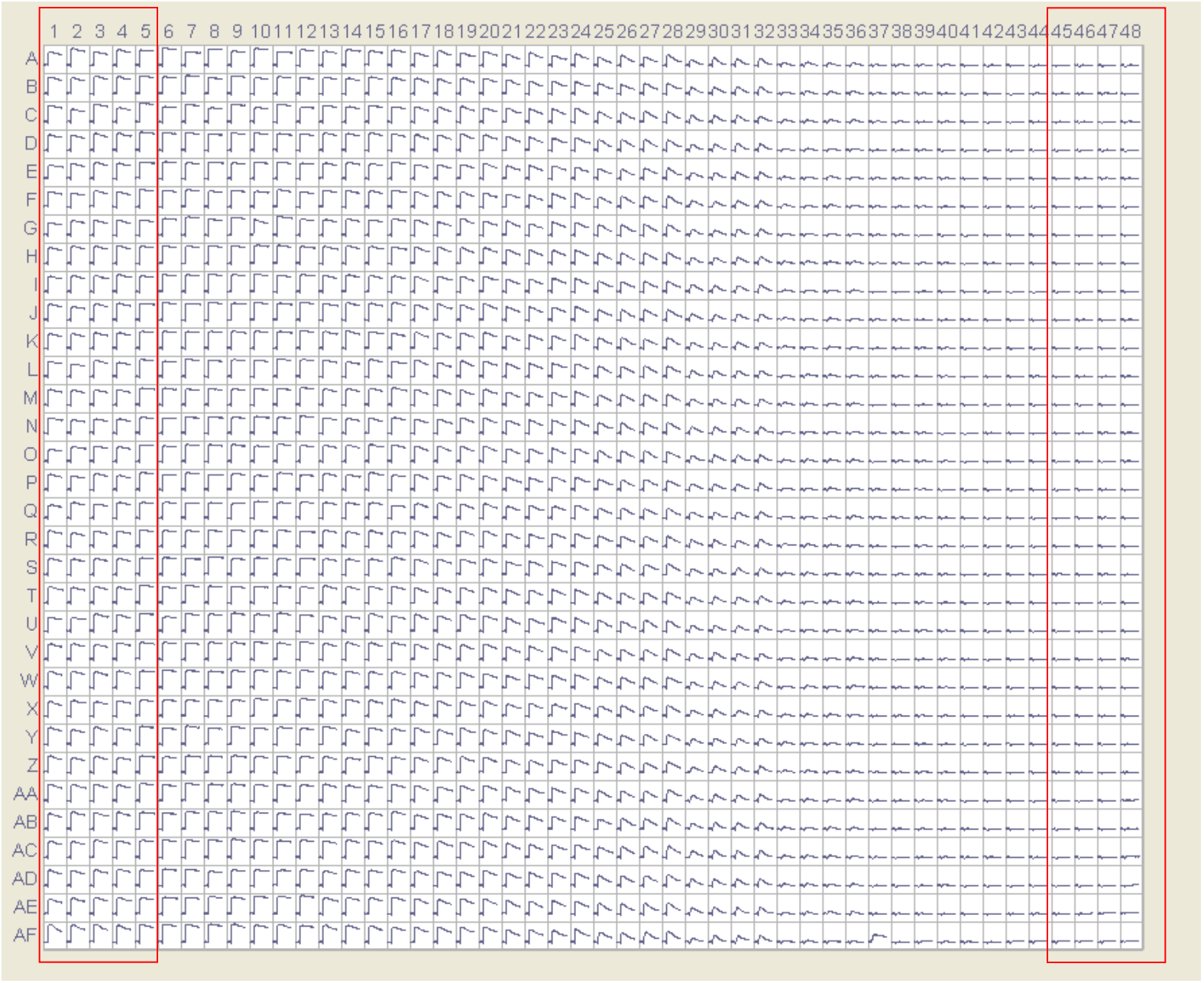
Z factors poor – reason = addition atrifact

Receptor “A”	Whole plate +ve		Whole plate -ve		
Whole plate	Mean	Std dev	Mean	Std dev	Z factor
Plate 1	197.0160655	31.53388	48.66897	26.83608	-0.18041
Plate 2	170.6461951	25.2911	53.79451	10.60334	0.078462
Plate 3	119.4877457	22.337	23.9505	6.351549	0.09914
Control wells					
Plate 1	196.2253066	25.4809			-0.06367
Plate 2	176.6495418	29.88158			0.011398
Plate 3	137.0638545	26.96953			0.116256

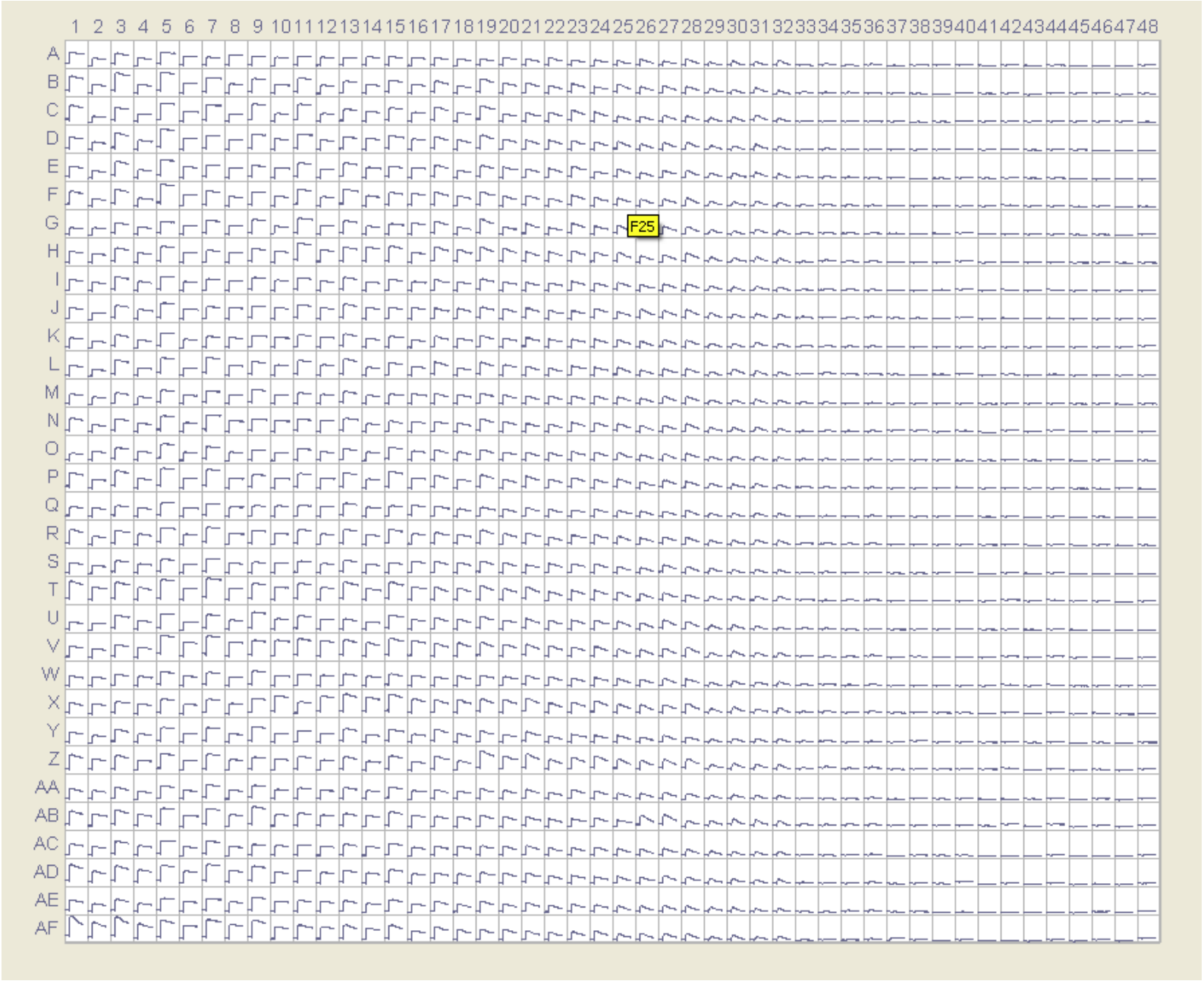
Method

- Isolated HEK cells, diluted to 2.0 million /ml and plated out into Greiner 1536 well low base plates using DW4 dispenser
- Incubate for 24 hrs
- Aspirate media (DW4), dispense 5ul loading buffer containing Fluo-4 and quencher (Brilliant Black) (normal conditions)
- Incubated for 45 mins @ 37^{o}C
- Dispense 1ul compound in aqueous DMSO
- Incubate for 15 mins @ 37^{o}C
- Read and dispensed 2ul of agonist to wells on Tetra

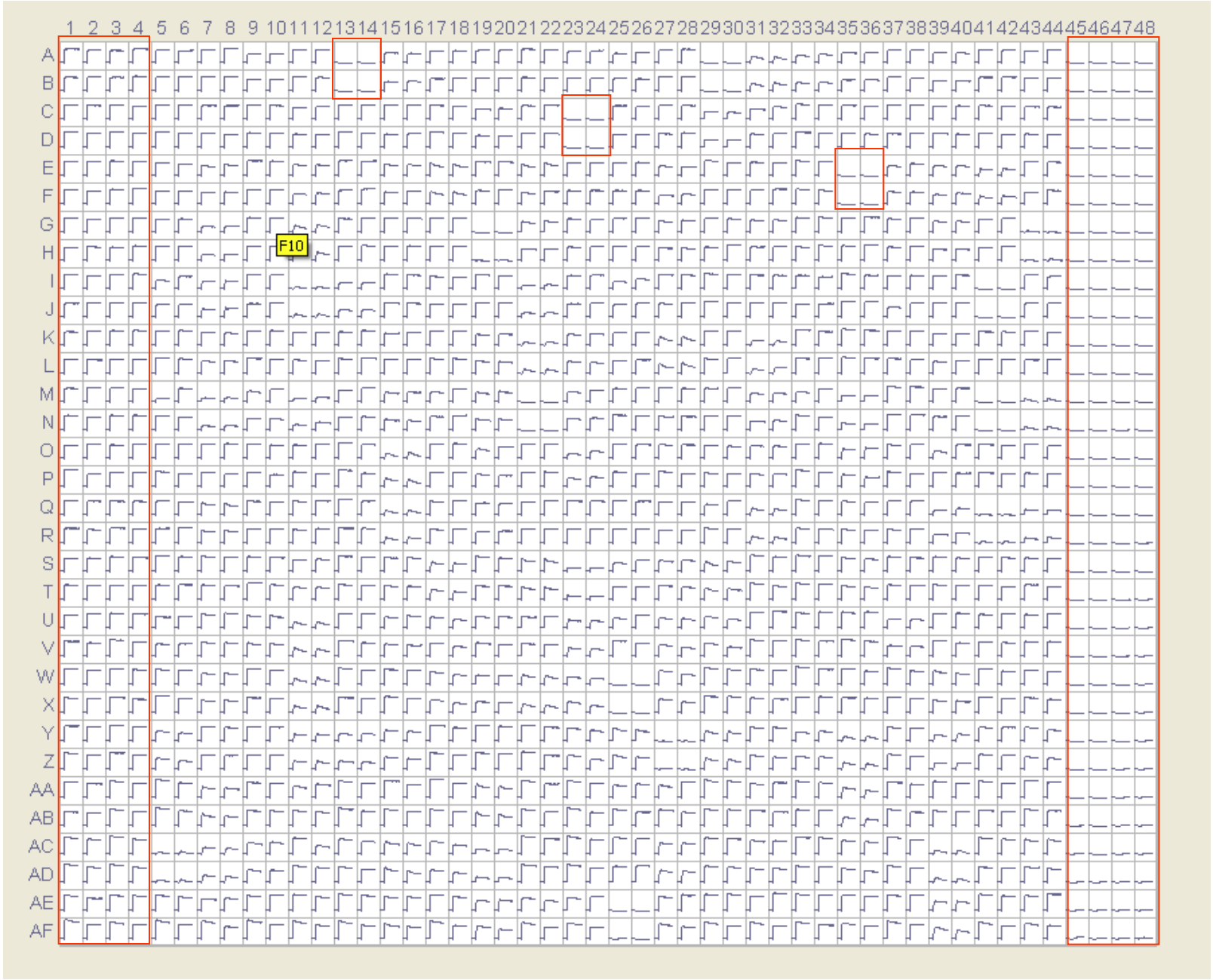
HEK-cells in Brilliant Black - Dose-Response Curve to Agonist



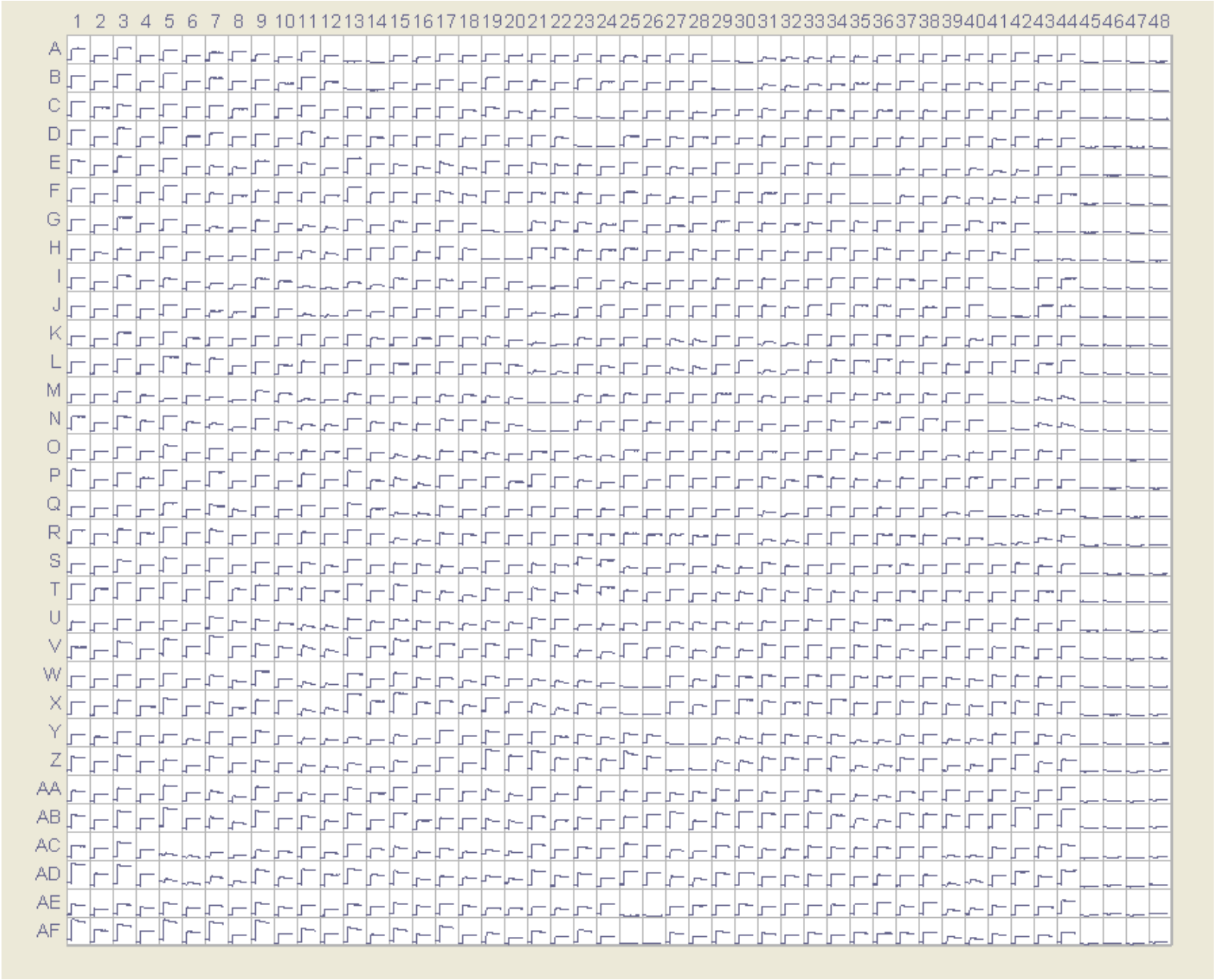
Uncorrected – NO Spatial Uniformity Correction



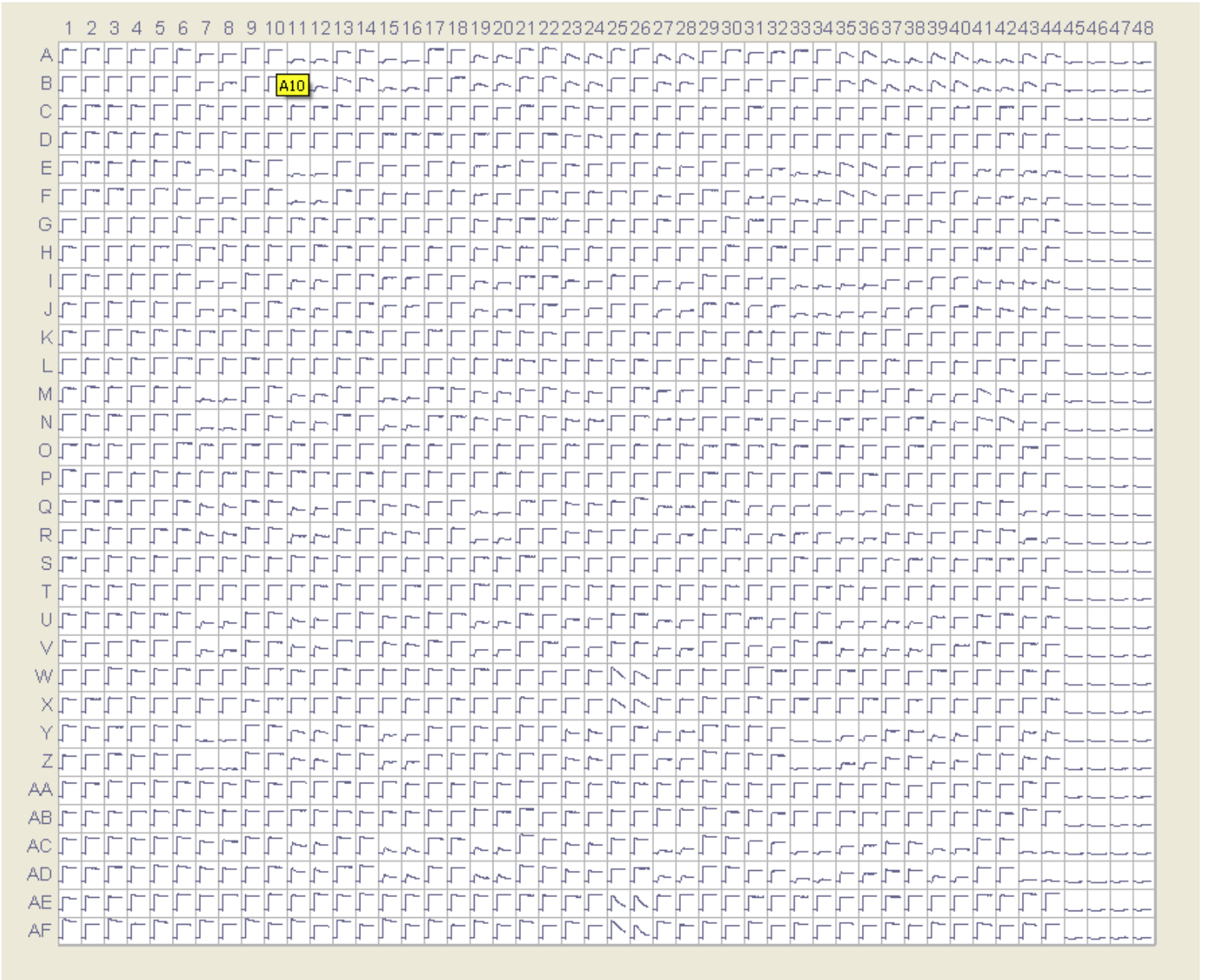
EC80 Across Plate plus Compound Addition in Quadruplicate (P1)



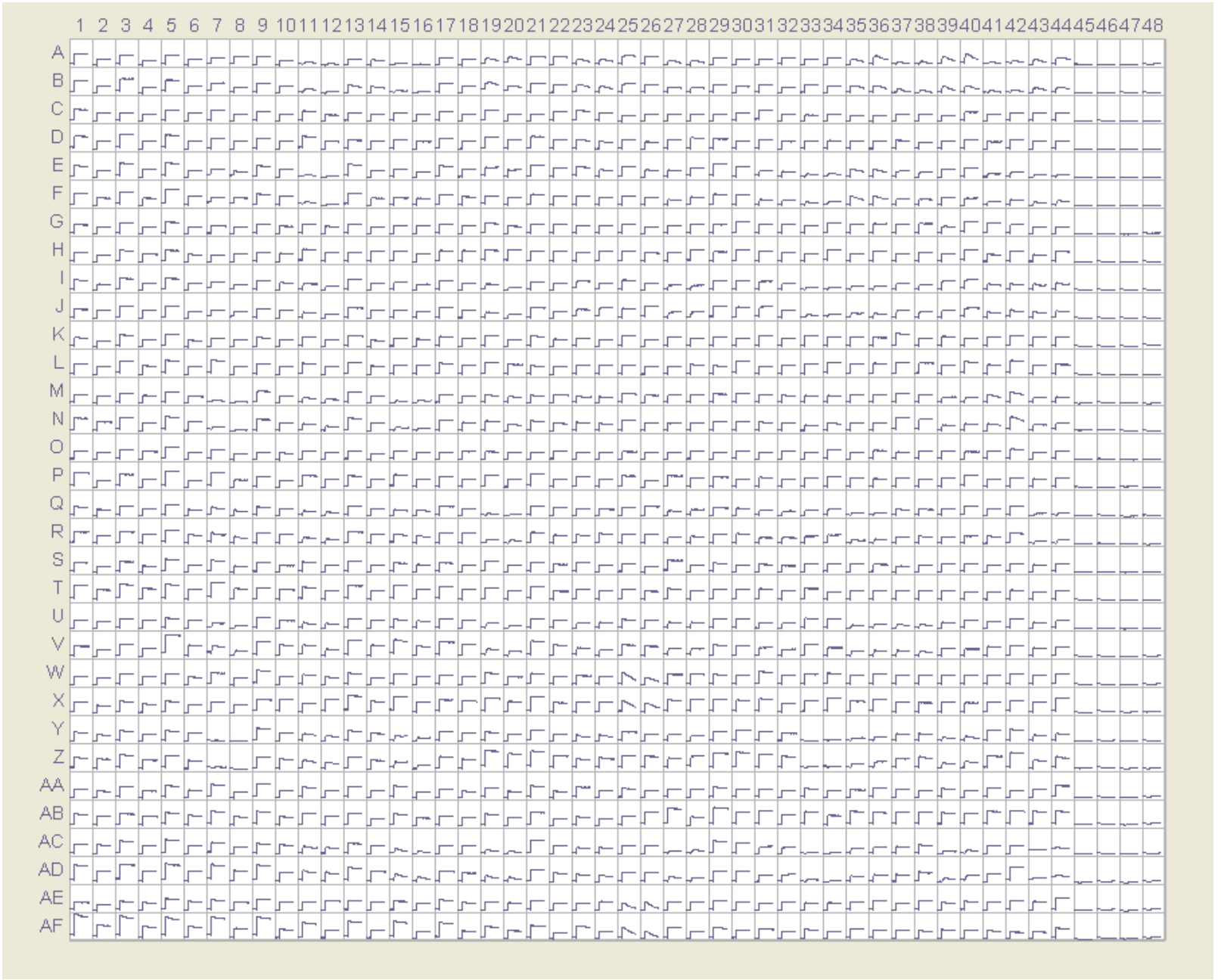
Uncorrected – NO Spatial Uniformity Correction



EC80 Across Plate plus Compound Addition in Quadruplicate (P2)



Uncorrected – NO Spatial Uniformity Correction



Technical Difficulties for HTS in 1536

- Aspirating ALL of the media
  - (DW-4 aspiration head needs to be re-engineered so that it can be adjusted to line it up with the 1536 well plate
- Dispensing the loading buffer without removing the cells
  - Need the 1536 well head on Tetra put on to another liquid handling platform to allow gentle addition of loading buffer. MDC need to develop and sell this or OEM to another supplier
- Dispensing compounds in a timely manner to all wells (1536 compared to 384)
  - Labcyte Echo 555 or off-line 384 to 1536 well dispensing head accurate at 1ul.
- Generating a 1536 well agonist plate
  - Too difficult to do manually, could quadruplicate from 384 but loose advantage of flexibility with control wells (EC50 curves etc). Need a single channel pipetting device to go from say microtubes to 1536 and that is fast

Summary - 1536

- Able to demonstrate calcium induced responses in human neutrophils to a number of ligands.
- Demonstrated;
  - Simple method
  - Good/excellent Z factors for 1536 well plates
  - Good correlation of pharmacology between compounds
  - excellent reproducibility in triplicate screening
- Practicalities of HTS with human neutrophils –
  - 2 donors/day x 200mls blood each
  - from each donor should get 400 – 500 million cells = 15-30 x 1536 well plates, equivalent to 60-120 x 384 well plates
- Also demonstrated 1536 format feasible for cell line work but problems with washing and loading buffer replacement make this approach less attractive at present

Conclusions

- Tetra is an excellent instrument for 1536 calcium work in human neutrophils – only criticism is price of 1536 well equipment

- DW4 good dispenser for plating cells and for aspirating spent media BUT not for dispensing loading buffer

- Echo 550 (Labcyte) excellent instrument for dispensing compounds into plates either into dry plate or on top of cells – also very quick for D/R curve generation

Thanks go to……

- Tracy Martin
- Kathy Dodgson
- Paul Hemsley
- Martin Coldwell