Side by side: An evaluation of 2D vs. 3D cell culture for High Throughput Screening in Drug Discovery

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- 3D cell culture has the potential to provide a **more physiologically relevant model** compared to standard tissue culture plastic.
- From a screening perspective the technology offers the possibility of **more predictive drug responses** but has an **increased cost**.
- The question: **is it possible** and, more importantly, **is it worthwhile** moving towards **screening in High Throughput using a 3D model**?

Reporter cell line assay and 3D Technology background.

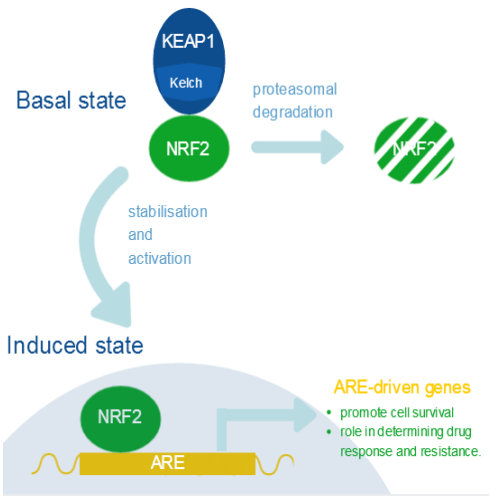


Figure 1. Nrf2 pathway. A549-ARE cell line have mutated KEAP1 so are constantly in induced state.

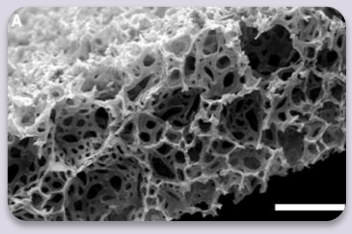
Oncology target Nrf2 investigated in 2D and 3D formats.

Nrf2 (nuclear factor-like 2) is a transcription factor that induces expression of genes bearing an antioxidant response element (ARE) in their regulatory regions **(Figure 1)**.

Mutations in the Kelch domain of KEAP1 have been reported in 40% of non small cell lung cancer cell lines. Mutations have been shown to disrupt repressor function of KEAP1 so leading to activation of the Nrf2 response pathway. (Namani et al., 2014)

Microtitre plates with Alvetex inert scaffold integrated into 384-well plates were evaluated as a potential 3D technology for HTS alongside traditional microtitre plates.

A549-ARE luciferase reporter cell line was used to develop aligned 2D + 3D assays: inhibitors of Nrf2 pathway identified by **incubation of cell line with compound** followed by detection of **luciferase activity** to measure transcriptional activity of Nrf2.



Alvetex Inert Scaffold
Highly porous polystyrene scaffold 200µm thick, heat welded to base of well. Regular-sized voids interconnected by pores allow cells to interact with neighbours. (Reinnervate, 2014)

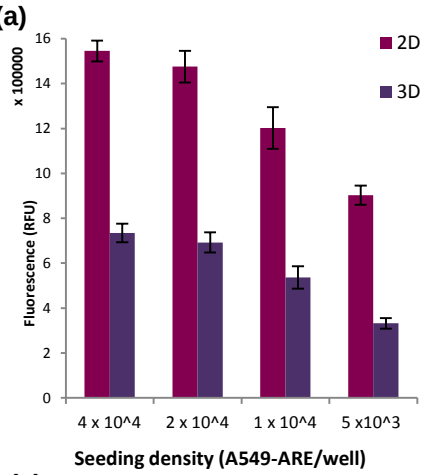
Optimisation of Nrf2-ARE assay in 2D and 3D platforms demonstrates comparable assay quality.

To select seeding density of A549-ARE on 3D plates, cell viability analysis carried out **(Figure 2a)**. Density was doubled for 3D plates to compensate for larger surface area.

To ensure comparable data, quality metrics for 2D and 3D plates obtained using luciferase read **(Figure 2b)**.

The developed workflow of the Nrf2-ARE assay method **(Figure 2c)** is suitable for automation.

(a)



(b)

	3D	2D
RZ' of QC plate	0.80	0.73
%CV of Max plate	9%	8%
DMSO tolerance	1%	1%

(c)

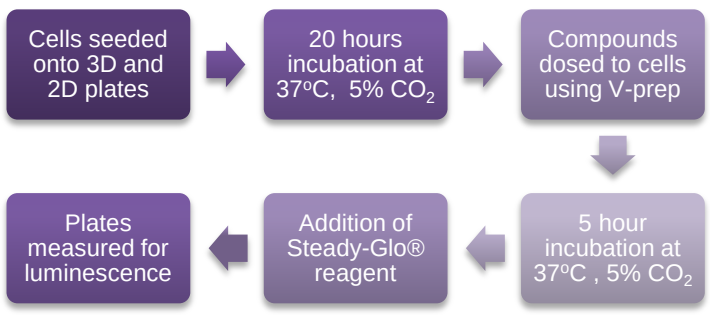


Figure 2. Optimisation for 3D plates. (a) Cell Viability measured using Cell Titer Blue for A549-ARE on 3D and standard 2D after 20 hours of growth. 1x10⁴/well used in 3D plates and 5x10³ in 2D plates (b) Comparable quality metrics found in reporter assay for 3D and 2D (c) Workflow developed for automated NRF2-ARE assay to enable 2D vs. 3D compound screen.

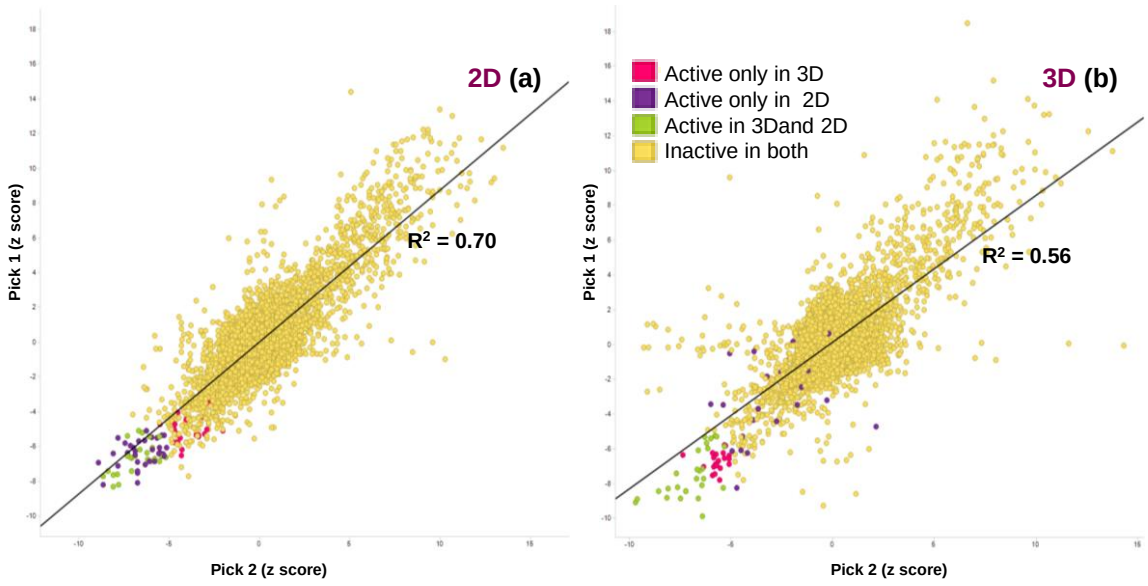
Identification of compounds with specific activity in 2D and 3D formats.

Single concentration screen at 10µM of 7000 compound diversity set screened in duplicate using Nrf2-ARE assay, run in parallel on tissue culture plastic (2D) and on Alvetex inert scaffold (3D) (Figure 3a/b).

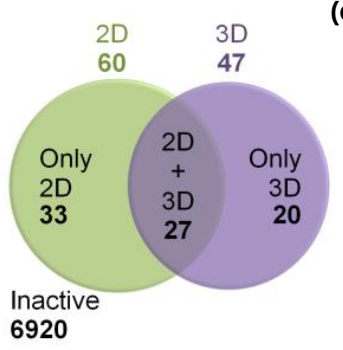
Duplicates are positionally different arrangements of compounds on plates and show good correlation. Robust Z' (0.67 2D; 0.79 3D) of QC plates demonstrates **assay is robust in 3D and 2D**. Actives identified using a statistical cut-off show **reduction in total hit rate when using 3D** plate (0.84% in 2D vs. 0.66% in 3D).

80 actives were identified, a number of compounds uniquely active in each format (Figure 3c).

Chemistry input determined that additional to singleton and pairs of compounds there were **distinct clusters** identifiable **(Figure 3d)**, three detected in both 3D and 2D, while two clusters only detected in 3D.



(c)



(d)

Cluster	Active in 3D	Active in 2D
1 known specific inhibitors	Y	Y
2 non specific ARE inhibitors	**Y**	N
3 non specific ARE inhibitors	**Y**	N
4 undefined action	Y	Y

Figure 3. Validation screen. Correlation of Pick 1&2 of compound set in (a) 2D plates and (b) 3D plates. Actives (ZS ≤ -5) highlighted. (c) Compound hits from validation set with cut-off of Z score of ≤-5 gives hit rate of 0.66% in 3D and 0.84% in 2D. (d) Structurally similar compound clusters identified in diversity set. Cluster 1 contains specific inhibitors of Keap1/Nrf2 pathway. Compounds in clusters 2 and 3 are structurally different to each other but are all inhibitors of ARE driven gene expression that may work via mechanism independent of Keap1 mutation. Cluster 4 compounds may function through any kinase in the Nrf2 pathway.

Actives confirmed and potency of compounds determined.

Concentration response analysis demonstrated no bias for potency between 2D and 3D: mean difference in $logIC_{50}$ is 0.02 and 97% of compounds <2S.D from mean **(Figure 4).**

Compounds found to have activity in only one format in single shot concentration screen were reproducible and showed low potency in CR for other format ($logIC_{50}$ ≤5.3).

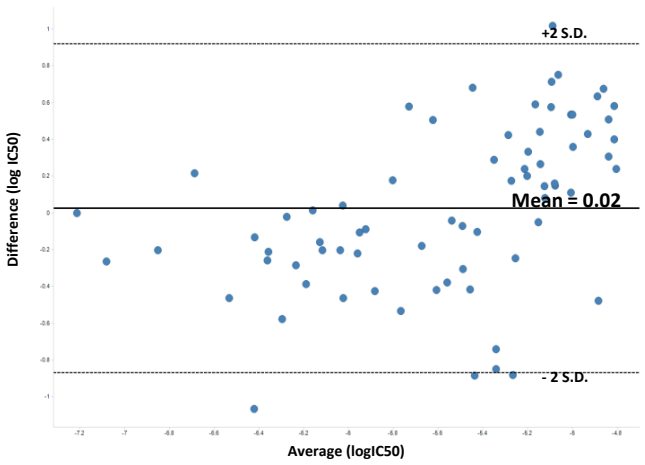


Figure 4. Bland-Altman for IC_{50} values of 80 identified actives

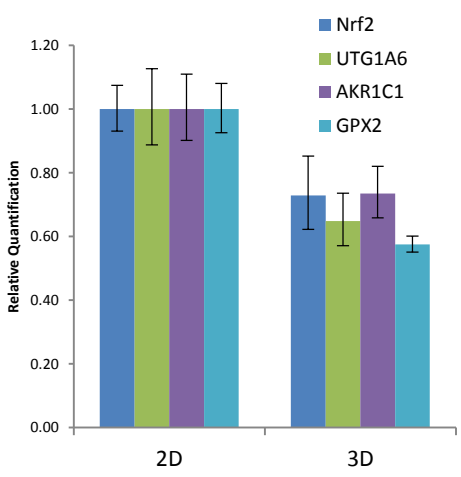


Figure 5. Expression of Nrf2 and downstream genes are downregulated on 3D.

Nrf2 and downstream genes downregulated in 3D

Gene expression of Nrf2 and three downstream target genes analysed using RTq-PCR. Difference in expression of genes supports evidence for differential compound response seen in validation screen data.

Conclusions: 3D cellular assay suitable for High Throughput Screening

- Unique clusters identified in this assay as active in 3D not seen in 2D
- Suggest alternative hits could be obtained in full screen
- Technology suitable to adapt to automation platforms
- More feasible cost and scale than current 3D alternatives
- Recommendations for improved design shared with manufacturer to develop suitability to HTS. These include well shape and lid type.

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AstraZeneca

References: Namani, A., Li, Y., Wang, X. and Tang, X. (2014). Modulation of NRF2 signaling pathway by nuclear receptors: Implications for cancer. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*. ; Reinnervate, (2014). Alvetex Product Formats. [online] Available at: http://reinnervate.com/why-alvetex/formats/