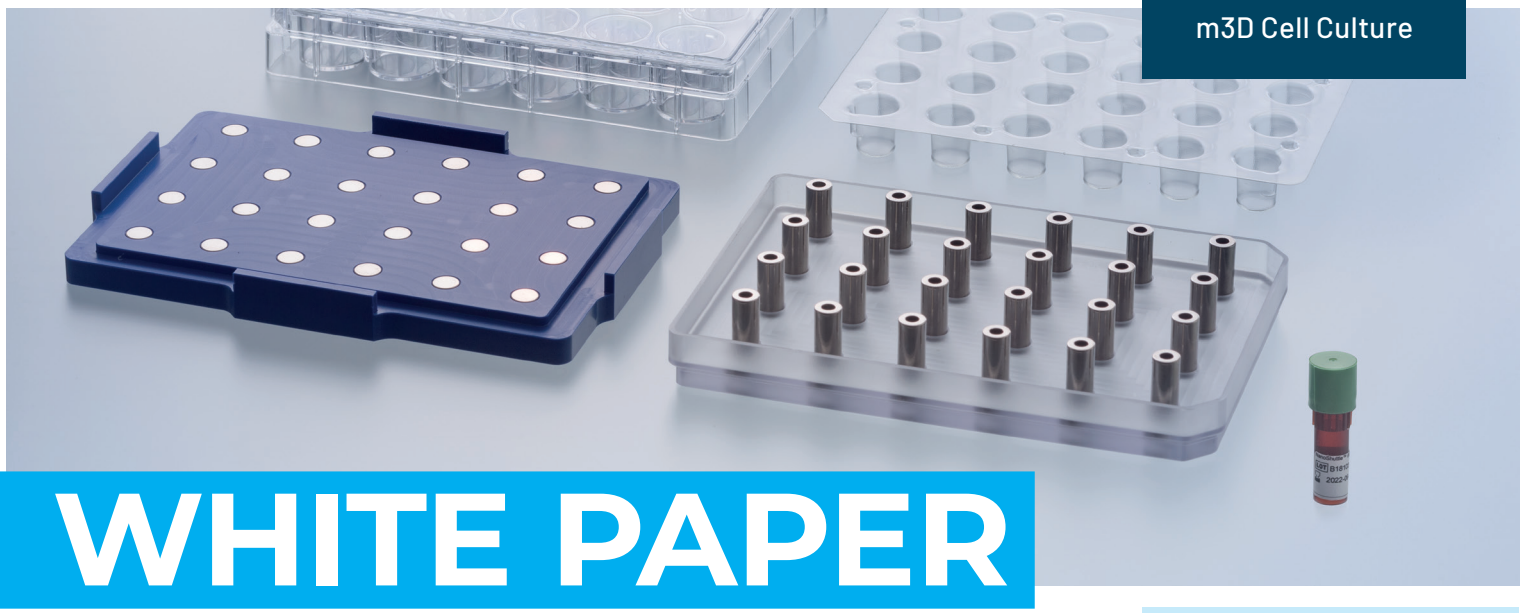

WHITE PAPER

TECHNICAL NOTES & APPLICATIONS FOR LABORATORY WORK

CONTENT

M3D CELL CULTURE: BIOCOMPATIBILITY OF NANOSHUTTLE-PL AND THE MAGNETIC FIELD

1/ INTRODUCTION

Biomedical research has gravitated towards three-dimensional (3D) cell culture in order to improve accuracy and validity over traditional two-dimensional (2D) monolayers. There are several 3D cell culture platforms on the market, none of them as easy in handling and innovative as the magnetic levitation and magnetic 3D bioprinting. The principle behind these methods is the magnetization of cells with a nanoparticle assembly, Nano-Shuttle-PL. Magnetized cells can then be aggregated by either: magnetic levitation, where cells are levitated by a magnet above the plate to aggregate at the liquid surface[1, 2]; or magnetic 3D bioprinting, where cells are rapidly aggregated at the bottom of a CELLSTAR® cell-repellent plate with a magnetic drive below the plate.[3] Once aggregated, these cells interact and self-assemble into a 3D culture, recreating an in vivo enviroment. The advantage of the m3D technology is the rapidity and reproducibility of the spheroid formation, the high quality optical analysis in flat bottom plates and the position control of the culture by the magnets.

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The NanoShuttle-PL as essential part of the magnetic 3D cell culture technology is responsible for the magnetisation of the cells. Once magnetized the cells can be aggregated by magnetic forces and theses cells interact and self-assemble into a culture that recreates in vivo environments. In addition the magnetic feature can be used holding the 3D cell culture in place while processing. A common concern is the potential for toxic or other adverse effects of NanoShuttle-PL. Various research with this platform from our lab and within independent studies of our users has shown so far no effect of NanoShuttle-PL on cell health or function. This application note will discuss in further detail the results demonstrating the biocompatibility of NanoShuttle-PL.

2/ WHAT IS NANOSHUTTLE-PL?

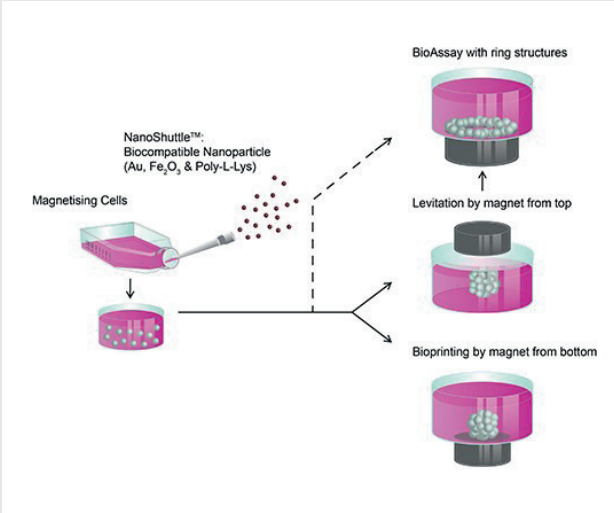

Figure 1: Schematic of magnetic levitation and magnetic 3D bioprinting.

Cells are magnetized by the binding of NanoShuttle-PL to the cells. NanoShuttle-PL is a nanoparticle assembly smaller than 50 nm in size consisting of gold, iron oxide, and poly-L-lysine (PLL).[1] Although NanoShuttle-PL is not itself an FDA-approved product for use in humans, the constituent components are themselves biocompatible.

Both iron oxide and PLL are recognized as safe by the FDA.[4,5] Gold nanoparticles have also been shown, in **"in vitro"** studies[6] and clinical trials[7], to not induce acute toxicity.[8] Taken altogether, NanoShuttle-PL should pose no toxicity concerns given its contents.

3/ HOW MAGNETIZATION WORKS

NanoShuttle-PL magnetizes cells by electrostatically attaching to cell membranes via PLL at a concentration of around 50 pg/cell.1 In this mechanism, NanoShuttle-PL non-specifically attaches to the membrane. The small amount of NanoShuttle-PL sparsely covers the cell, instead of the whole membrane, giving a pepped -like appearance.

Figure 2: **Human pulmonary fibroblasts** before (left) and after (right) magnetization with NanoShuttle-PL. Scale bar = 100 µm.[11]

The amount is also enough to attract cells with mild magnetic forces. With fields of around 50-500 G used in magnetic levitation or magnetic 3D bioprinting, forces of 30 pN/cell are exerted on the cells to attract them. Once magnetized, the NanoShuttle-PL remains on the cell membrane for at most 7-8 days. This was demonstrated with another version of NanoShuttle-PL consisting of bacteriophage instead of PLL.[1]

Transmission electron microscopy (TEM) showed that NanoShuttle-PL was localized with cells at day 0, but by day 8, released off the cells into the extracellular matrix (ECM). Thus, by then, NanoShuttle-PL will no longer be attached to the cell, but by being retained in the ECM, it will continue to magnetize the culture, and allow for the use of the magnets to hold cultures down during processing.

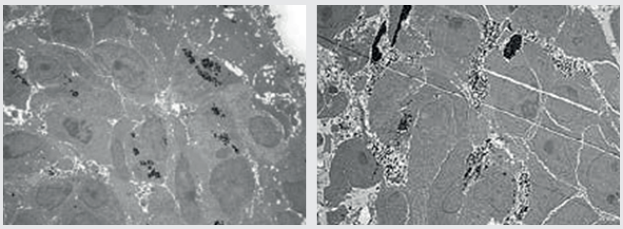

Figure 3: TEM images of 3D cultures of glioblastoma. At day 0 (left), NanoShuttle-PL localizes with the cell, but by 7 days (left), NanoShuttle-PL releases off of the cell and aggregates in the ECM.[1]

4/ VIABILITY AND PROLIFERATION

Through numerous experiments, we have come to the understanding that NanoShuttle-PL does not have any effect on cell viability or proliferation. Using common biochemical assays for viability, such as CellTiter-Blue (Promega) and the MTT assay, we have shown that adding NanoShuttle-PL to cell monolayers does not reduce viability.

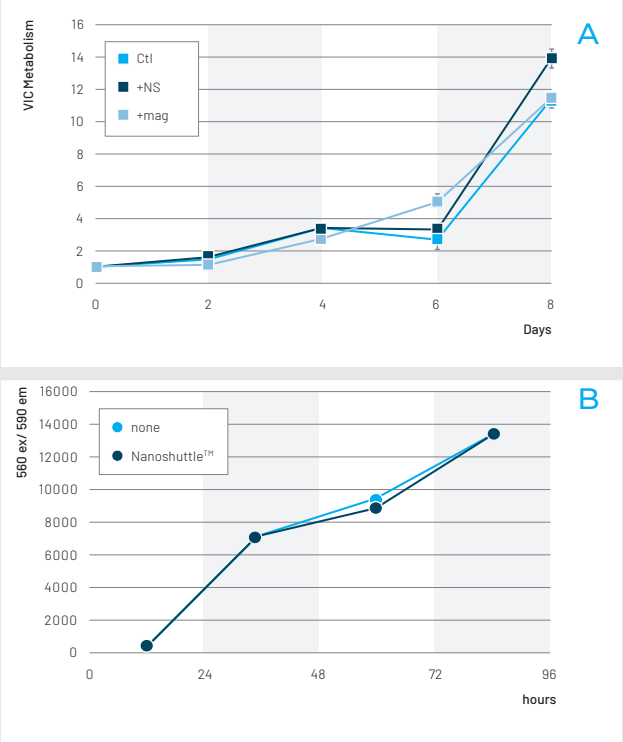

Figure 4: The effect of NanoShuttle-PL and the magnetic field on viability of VICs11 (A) and the effect of NanoShuttle-PL on 3T3 viability (B) as measured by the MTT assay on monolayers.9 Error bars represent standard deviation.

The cell types tested include: 3T3 murine embryonic fibroblasts[9]; primary human pulmonary fibroblasts (PF), pulmonary endothelial cells (PEC), bronchial epithelial cells (BEpiC) and tracheal smooth muscle cells (SMC)[10]; and also primary porcine valvular interstitial cells (VIC) and endothelial cells (VEC).[11]

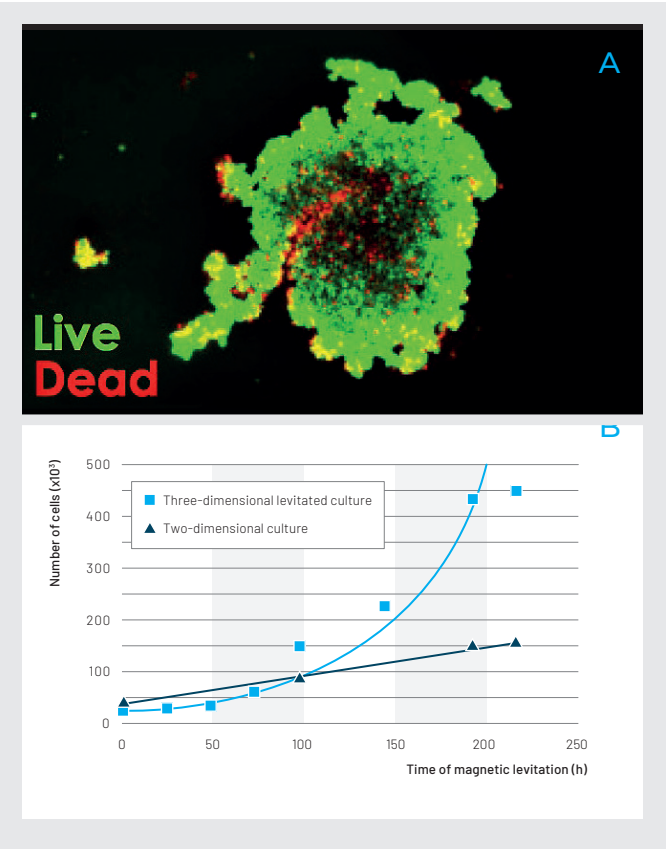

Figure 5: (A) Viability of spheroids of HepG2 hepatocellular carcinoma cells as measured by live/dead staining (live = green, dead = red).

(B) Growth of LN-229s in magnetic levitation (light blue) and in monolayers (dark blue).[1] Scale bar = 500 µm.

Aggregation into spheroids also does not alter cell viability. Cells within 3D cultures have been shown to be both viable[12] and proliferative with positive staining of Ki67.10 Moreover, viability is linearly correlated with starting cell number, demonstrating that cells are not dying when immediately aggregated. In fact, we showed that LN-229 glioblastoma proliferate faster in 3D culture than they do in monolayers.[1, 13, 14] Overall, our results demonstrate that there is no toxic effect of NanoShuttle™ on cell viability and proliferation.

5/ PHENOTYPE

In general, we have found that broad cell phenotypes and functions are preserved in cells with exposure to NanoShuttle-PL or in 3D. This includes: automatic beating in cardiomyocytes; CYP450 synthesis in hepatocytes; adipogenic differentiation potential of pre-adipocytes[9]; positive staining for Ecadherin and cytokeratin in epithelial cells[10, 15]; and extracellular matrix (ECM) remodeling by fibroblasts and smooth muscle cells.[10, 11]

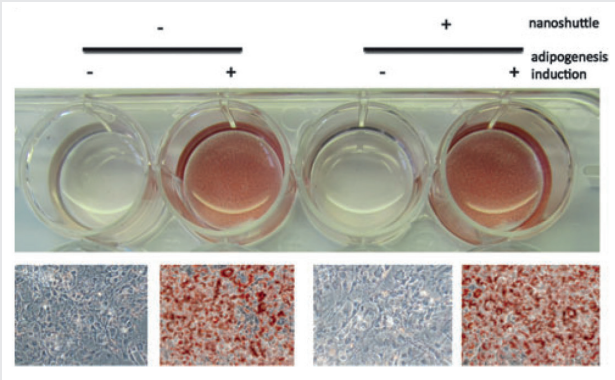

Figure 6: Lipid droplet accumulation in 3T3-L1 fibroblasts with (right) and without (left) NanoShuttle-PL, and with and without adipogenesis induction, as measured by Oil Red O staining (bottom, red). There was no visible effect of NanoShuttle-PL on differentiation.[9]

We also demonstrated with flow cytometry that LN-229s carry the same phenotype in 3D as they do in 2D as seen by similar PD-L1 expression between environments.

	Isotype MFI	Signal MFI	Signal Ratio
2D w/ NS	169	393	2.3
2D w/o NS	174	380	2.2
3D w/ NS	155	311	2.0
3D w/o NS	134	290	2.2

Table 1: PD-L1 expression.
Mean fluorescence intensities (MFI) of PD-L1 expression and signal ratio in LN-229 glioblastoma as measured with flow cytometry. Data acquired in collaboration with Intellicyt (Albuquerque, NM).

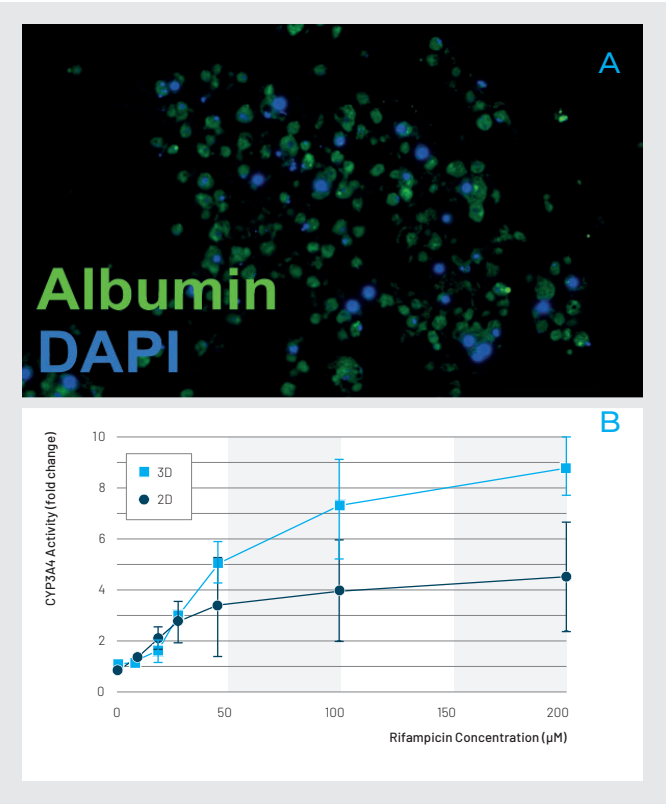

Figure 7: Maintenance of phenotype in 3D.
(A) Spheroids of primary hepatocytes (BioreclamationIVT, Baltimore, MD) stained positively for albumin (green) demonstrating their phenotype.
(B) CYP3A4 induction in induced pluripotent stem cell derived (iPS-) hepatocytes in spheroids (black) and monolayers (red) in response to rifampicin, where a higher induction of CYP3A4 activity was found in 3D compared to 2D. Scale bar = 50 μm. Error bars represent standard deviation.

The degree to which phenotypes are present vary based on cell type. For example, with hepatocytes, they showed a higher baseline activity of CYP450 in spheroids than they did in monolayers.

On the other hand, VICs displayed a quiescent phenotype compared to their activated phenotype in monolayers.[11] These changes in phenotype are likely due to the unattachment of the cells to a stiff substrate. Overall, NanoShuttle-PL alone does not affect phenotype.

6/ INFLAMMATORY STRESS

NanoShuttle-PL has also been shown not to induce any inflammatory stress in cells. When lung cells (BEpiC, SMC, PF, PEC) were exposed to NanoShuttle-PL, no significant difference in interleukin (IL)-6 and IL-8 production were found aside from IL-6 production in SMCs.[10] This suggests that NanoShuttle-PL does not promote inflammatory.

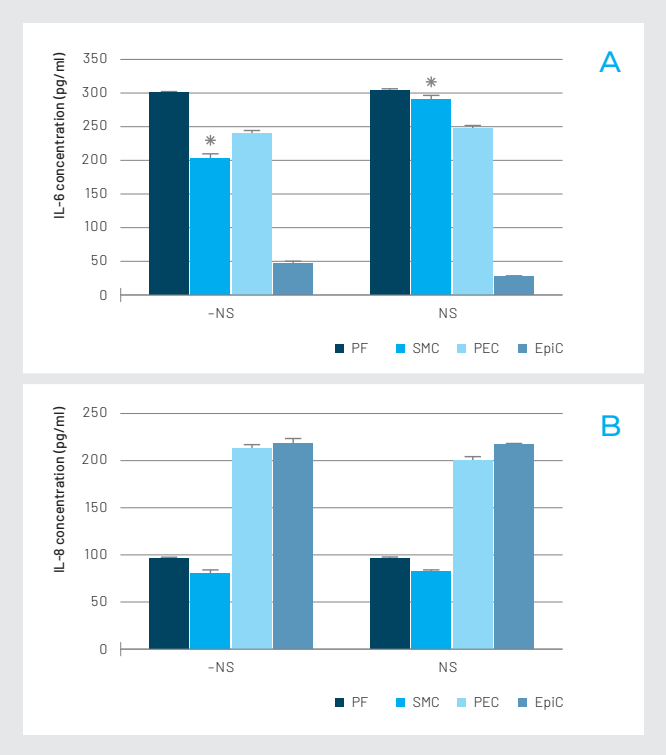

Figure 8: Effect of NanoShuttle-PL (NS) on IL-6 (A) and IL-8 production in cultures of PFs, SMCs, PECs, and BEpiCs after 6 h of exposure. Error bars represent standard deviation. *: $p < 0.05$ between groups.

7/ OXIDATIVE STRESS

We have also shown that NanoShuttle-PL does not induce oxidative stress. A higher gene expression of endothelial nitric oxide synthase (eNOS) was found in 3D cultures with VECs compared to monolayers, suggesting that these cells are ably producing NO to maintain a healthy oxidative environment.[11] These results suggest that in spite of the presence of NanoShuttle-PL, VECs in 3D are more able to regulate oxidative stress.

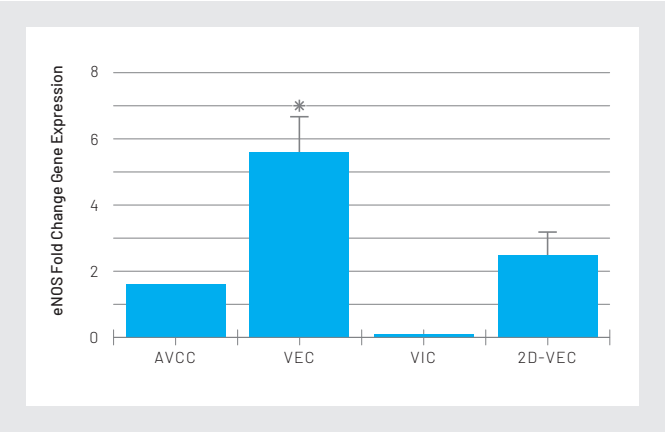

Figure 9: Fold changes in eNOS gene expression by VECs and VICs in 3D and 2D relative to an aortic valve co-culture (AVCC) of the two cell types. VECs in 3D expressed significantly higher amounts of eNOS compared to VECs in 2D.* : $p < 0.05$ compared to 2D. Error bars represent standard deviation.

8/ COMPARATIVE GENOMIC HYBRIDIZATION

To see if NanoShuttle-PL affected DNA, we used comparative genomic hybridization (CGH) to observe any changes in copy number as a result of incubation with NanoShuttle-PL. In doing so, we found no alterations in copy number as a result of exposure to NanoShuttle-PL in monolayers of PFs. These results demonstrate that NanoShuttle-PL does not lead to genomic instability. Taken altogether, we have demonstrated through experimentation that NanoShuttle-PL has no toxic or deleterious effect on cell health and function.

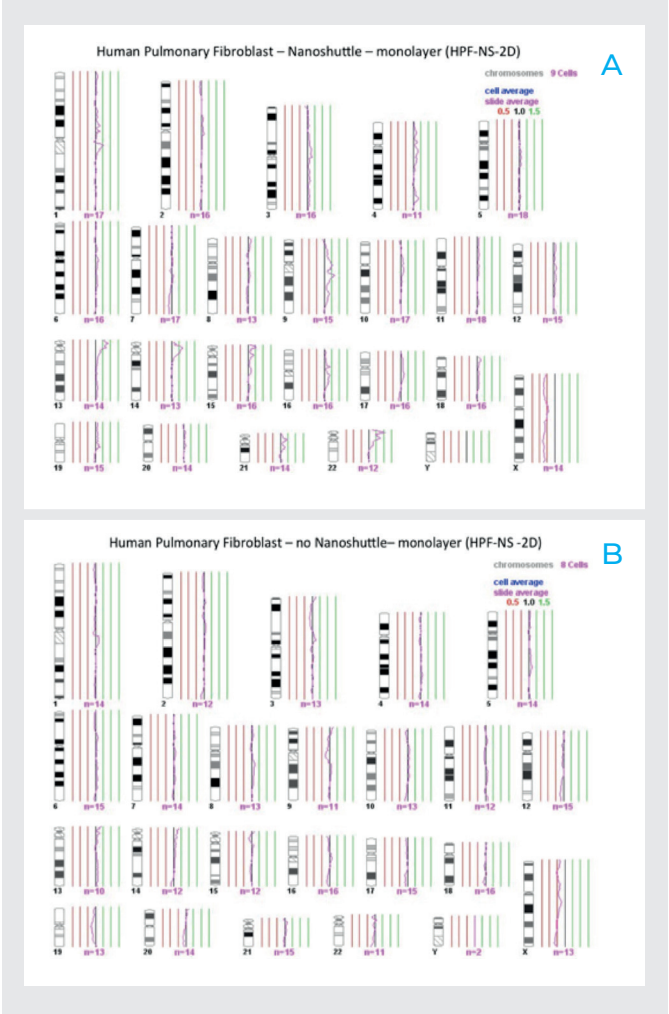

Figure 10: CGH profile of PFs in monolayers with (A) or without (B) NanoShuttle-PL. The bars graph the gains (B) and losses (A) of each chromosome relative to a normal reference genome. The loss in chromosome X can be attributed to the fact that PFs were donated from males.

9/ PDX COMPARISON – MORPHOLOGY, DOSE RESPONSE, AND GENOMIC PROFILE

Comparison of in vivo PDX vs. in vitro magnetic 3D bioprinted showed virtually no difference in morphology, protein expression, and coding genes or gene expression. Dose response comparison of vitro 3D vs. in vivo PDX compounds showed equivalent results for 8 compounds.[16]

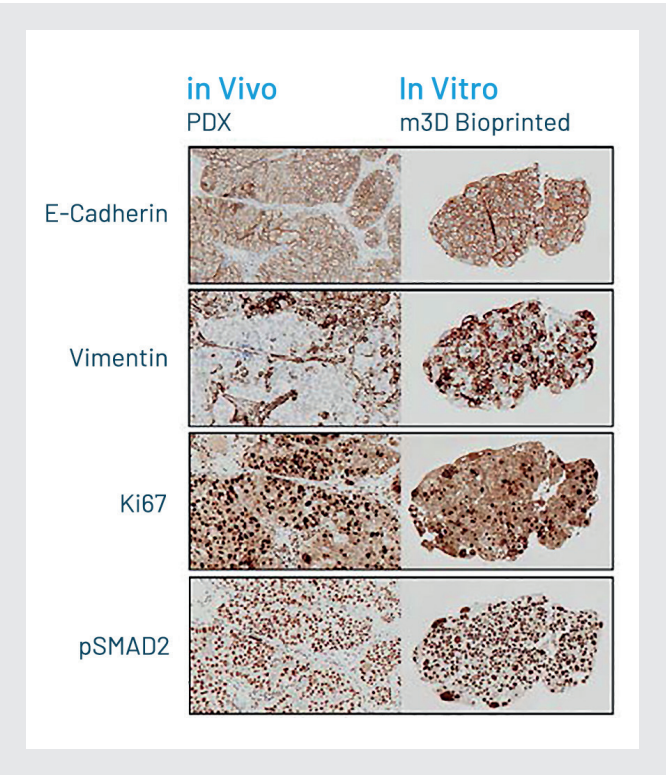

Figure 11: **Lipid droplet accumulation in 3T3-L1 fibroblasts** with (right) and without (left) NanoShuttle-PL, and with and without adipogenesis induction, as measured by Oil Red O staining (bottom, red). There was no visible effect of NanoShuttle-PL on differentiation.[9]

10/ HIGH-THROUGHPUT SCREENING

Magnetic 3D bioprinting provides a key strategic advantages, while NanoShuttle-PL shows no effect in dose response when compared with controls (no NanoShuttle-PL). NanoShuttle-PL shows to be bioinnert while M3D provides a rapid, relatively easy, and reproducible method for 3D cell-culture high throughput, including key points[17]:

- validated 384- and 1536-well magnetic 3D bioprinting systems as a powerful tool for HTS.
- homogeneous HTS organoid-based assay.
- standardized using well known anti-cancer agents against four patient-derived pancreatic cancer KRAS mutant associated primary cell lines.
- screen included cancer associated fibroblasts (CAF).
- cytotoxicity screen with a library of 3300 FDA approved drugs.
- ex vivo clinically relevant 3D tumor model can be rapidly adapted and used for large scale drug screening.
- Compared 3D vs. 2D HTS of 3,300 compound FDA library (FDA approved compounds) o2D showed many more active compounds which should not, "false positives" result o 3D identified compound that is currently undergoing clinical trial for pancreatic cancer, 2D did not.
- HTS done with flat bottom plates in 384- and 1536-well

„This work demonstrates a faster and more physiologically relevant cost-efficient drug discovery process, which ultimately will aid in avoiding possible false positives and improving the accuracy"

said Drs. Timothy Spicer and Louis Scampavia, leaders of this project from The Scripps Research Institute.

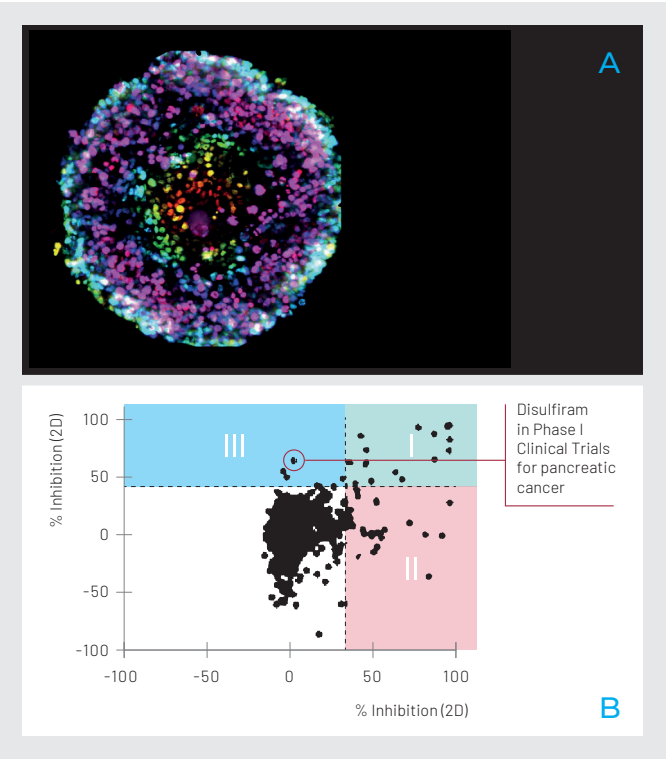

Figure 12: Effect of NanoShuttle-PL (NS) on IL-6 (A) and IL-8 production in cultures of PFs, SMCs, PECs, and BEpiCs after 6 h of exposure. Error bars represent standard deviation. *: $p < 0.05$ between groups.

4/ ORDERING INFORMATION

Magnetic 3D Cell Culture Products

Item No.	Description	Content kit
657841	NanoShuttle-PL	600 µl vials of NanoShuttle-PL (1)
657843	NanoShuttle-PL 3-pack	600 µl vials of NanoShuttle-PL (3)
657846	NanoShuttle-PL 6-pack	600 µl vials of NanoShuttle-PL (6)
657852	NanoShuttle-PL 12-pack	600 µl vials of NanoShuttle-PL (12)
657840	6 Well Bio-Assembler Kit	Levitation Drive, Holding Drive, NanoShuttle-PL (2 Vials), 6 well cell culture multiwell plates (2 x 657970) and 6 Well Intermediate lid (2 x 657825) with cell-repellent surface
662840	24 Well Bio-Assembler Kit	Levitation Drive, Holding Drive, NanoShuttle-PL (2 Vials), 24 well cell culture multiwell plates (2 x 662970) and 24 Well Intermediate lid (2 x 662825) with cell-repellent surface
655840	96 Well Bioprinting Kit, clear	NanoShuttle-PL (3 vials), Spheroid Drive, Holding Drive, 96 well cell culture microplates (clear) with cell-repellent surface (2 x 655970)

Magnetic 3D Cell Culture Products

Item No.	Description	Content kit
655841	96 Well Bioprinting Kit, black, µClear®	NanoShuttle-PL (3 vials), Spheroid Drive, Holding Drive, 96 well cell culture microplates (black, µclear®) with cell-repellent surface (2 x 655976-SIN)
655846	96 Well BiO Assay Kit	NanoShuttle-PL (3 vials), 6 Well Levitation Drive, 96 Well Spheroid, Holding and Ring Drive, 6 Well Intermediate lid (2 x 657825) with cell-repellent surface, 96 Well Deep Well Plate, 6 Well cell culture multiwell plates with cell-repellent surface (2 x 657970), 96 Well cell culture microplates (clear) with cell-repellent surface (2 x 655970)
781840	384 Well Bioprinting Kit, clear	NanoShuttle-PL (2 vials), Spheroid Drive, Holding Drive, 384 well cell culture microplates (clear) with cell-repellent surface (2 x 781970)
781841	384 Well Bioprinting Kit, black, µClear®	NanoShuttle-PL (2 vials), Spheroid Drive, Holding Drive, 384 well cell culture microplates (black, µClear®) with cell-repellent surface (2x 781976-SIN)
781846	384 Well BiO Assay Kit	NanoShuttle-PL (2 vials), 6 Well Levitation Drive, 384 Well Spheroid and Holding Drive, 6 Well Intermediate lid (2 x 657825) with cell-repellent surface, 96 Well Deep Well plate, 6 Well cell culture multiwell plates with cell-repellent surface (2 x 657970), 384 Well cell culture microplates (clear) with cell-repellent surface (2 x 781970)

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Greiner Bio-One GmbH Frickenhausen, Germany
PHONE +49 7022 948-0 / **FAX** +49 7022 948-514 / **E-MAIL** info@de.gbo.com

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