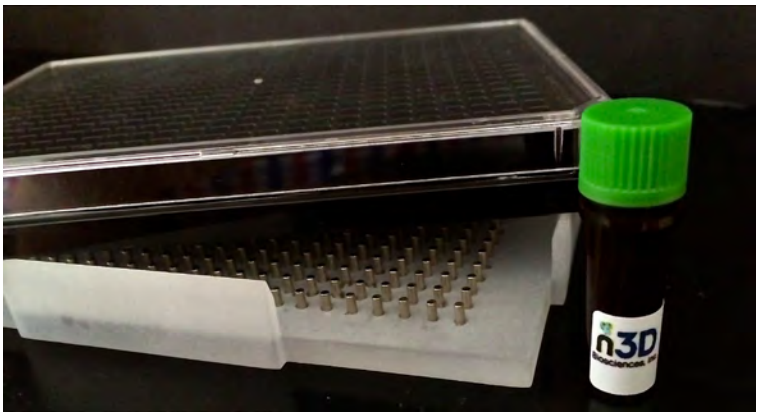
Biocompatibility of NanoShuttle™ and the magnetic field in magnetic 3D bioprinting

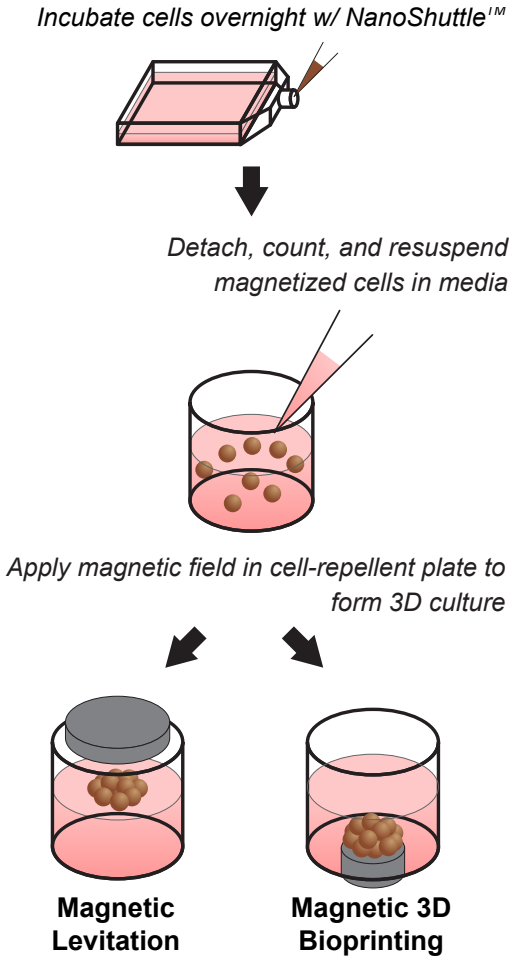
Biomedical research has gravitated towards three-dimensional (3D) cell culture in order to improve accuracy over traditional two-dimensional (2D) monolayers. There are several 3D cell culture platforms on the market, and among all of them are **magnetic levitation** and **magnetic 3D bioprinting**. The principle behind these methods is the magnetization of cells with a nanoparticle assembly, **NanoShuttle™**. Magnetized cells can then be aggregated by either: **magnetic levitation**, where cells are levitated by a magnet above the plate to aggregate away from a stiff substrate[1,2]; or **magnetic 3D bioprinting**, where cells are rapidly aggregated at the bottom of a **CELLSTAR®** cell-repellent plates with a magnetic drive below the plate.[3]



3D Bioprinting Kit with NanoShuttle™ and CELLSTAR® cell-repellent plates

Once aggregated, these cells interact and self-assemble into a culture that recreates *in vivo* environments. The advantage of this 3D cell culture platforms over others is the utility of the magnetic forces in both rapidly forming cultures, as well as holding them in place while processing.

A common concern is the potential for toxic or other adverse effects of NanoShuttle™. Research from our lab and our users with this platform has shown no effect of NanoShuttle™ on cell health or function. This application note will discuss in further detail our results demonstrating the biocompatibility of NanoShuttle™.



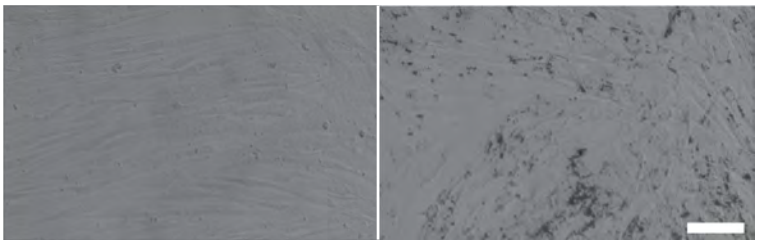
Schematic of magnetic levitation and magnetic 3D bioprinting

What is NanoShuttle™?

Cells are magnetized by the binding of NanoShuttle™ to the cells. NanoShuttle™ is a nanoparticle assembly smaller than 50 nm in size consisting of gold, iron oxide, and poly-L-lysine (PLL).[1] Although NanoShuttle™ 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™ should pose no toxicity concerns given its contents.

How Magnetization Works

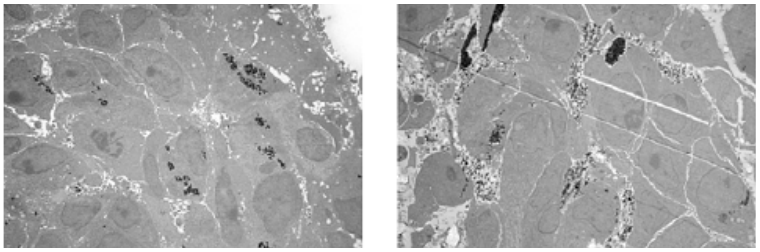
NanoShuttle™ magnetizes cells by electrostatically attaching to cell membranes via PLL at a concentration of around 50 pg/cell.[1] In this mechanism, NanoShuttle™ non-specifically attaches to the membrane. The small amount of NanoShuttle™ sparsely covers the cell, instead of the whole membrane, giving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



Human pulmonary fibroblasts before (left) and after (right) magnetization with NanoShuttle™. 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.

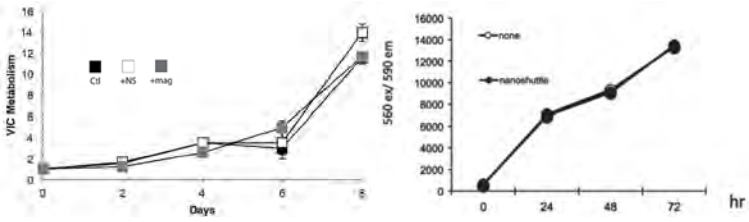
Once magnetized, the NanoShuttle™ remains on the cell membrane for at most 7-8 days. This was demonstrated with another version of NanoShuttle™ consisting of bacteriophage instead of PLL.[1] Transmission electron microscopy (TEM) showed that NanoShuttle™ was localized with cells at day 0, but by day 8, released off the cells into the extracellular matrix (ECM). Thus, by then, NanoShuttle™ 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.



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

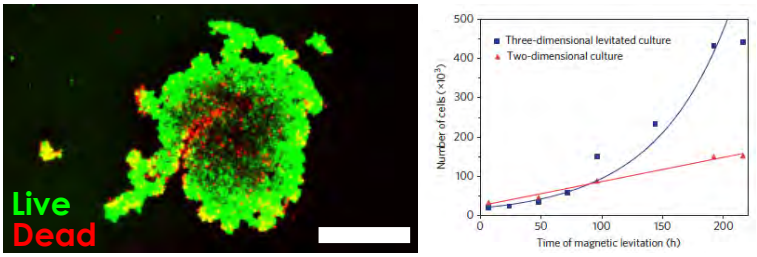
Viability and Proliferation

Through numerous experiments, we have come to the understanding that NanoShuttle™ 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™ to cell monolayers does not reduce viability. 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]



The effect of NanoShuttle™ and the magnetic field on viability of VICs[11] (left) and the effect of NanoShuttle™ on 3T3 viability (right) as measured by the MTT assay on monolayers.[9] Error bars represent standard dviation

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]

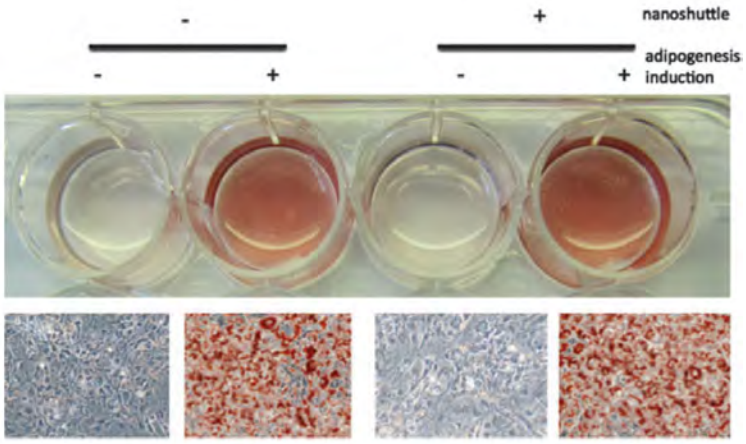


(Left) Viability of spheroids of HepG2 hepatocellular carcinoma cells as measured by live/dead staining (live = green, dead = red). (Right) Growth of LN-229s in magnetic levitation (blue) and in monolayers (red).[1] Scale bar = 500 µm.

Overall, our results demonstrate that there is no toxic effect of NanoShuttle™ on cell viability and proliferation.

Phenotype

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



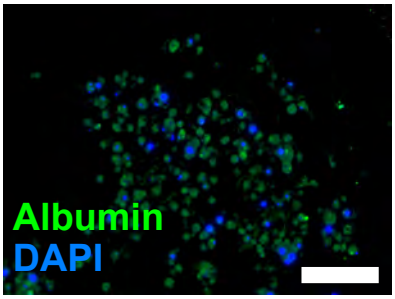
Lipid droplet accumulation in 3T3-L1 fibroblasts with (right) and without (left) NanoShuttle™, and with and without adipogenesis induction, as measured by Oil Red O staining (bottom, red). There was no visible effect of NanoShuttle™ on differentiation.[9]

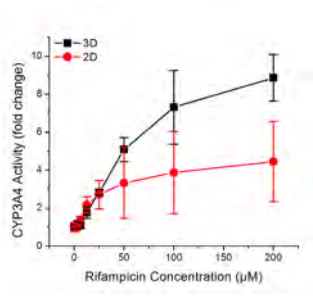
We also demonstrated with flow cytometry that LN-229s carry the same phenotype in 3D as they do in 2D as seen by similar PD-L1 expression between environments.

	PD-L1 expression		
	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

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).

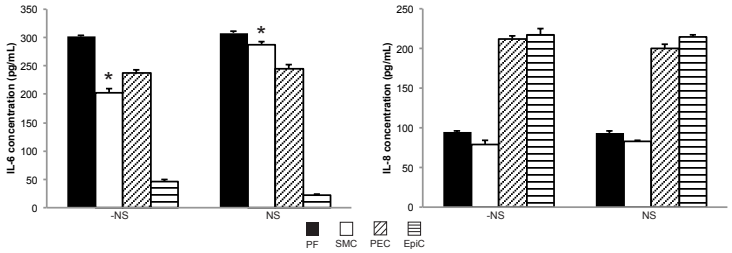
The degree to which phenotypes are present vary based on cell type. For example, with hepatocytes, they showed a higher baseline activity of CYP450 in spheroids than they did in monolayers. On the other hand, VICs displayed a quiescent phenotype compared to their activated phenotype in monolayers.[11] These changes in phenotype are likely due to the unattachment of the cells to a stiff substrate. Overall, NanoShuttle™ alone does not affect phenotype.





Maintenance of phenotype in 3D. (Left) Spheroids of primary hepatocytes (BioreclamationIVT, Baltimore, MD) stained positively for albumin (green) demonstrating their phenotype. (Right) 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.

Inflammatory Stress

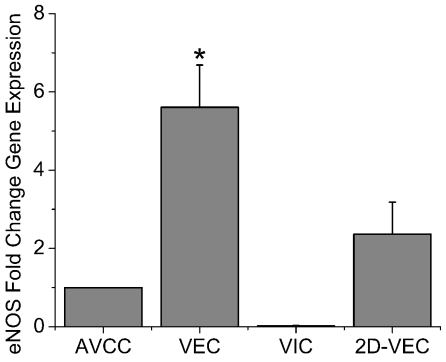


Effect of NanoShuttle™ (NS) on IL-6 (left) and IL-8 production in cultures of PFs, SMCs, PECs, and BEpiCs after 6 h of exposure. Error bars represent standard deviation. *: p < 0.05 between groups.

NanoShuttle™ has also been shown not to induce any inflammatory stress in cells. When lung cells (BEpiC, SMC, PF, PEC) were exposed to NanoShuttle™, 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™ does not promote inflammatory stress.

Oxidative Stress

We have also shown that NanoShuttle™ 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™, VECs in 3D are more able to regulate oxidative stress.



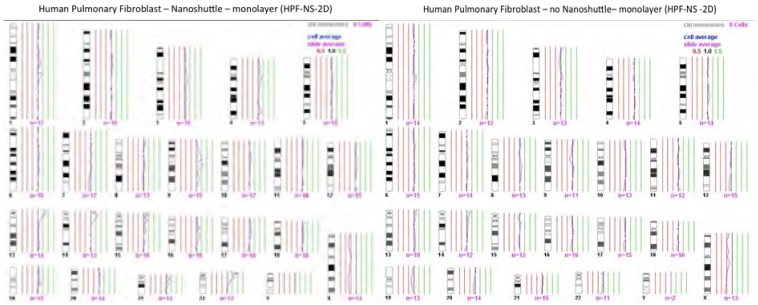
Fold changes in eNOS gene expression by VECs and VICs in 3D and 2D relative to an aortic valve co-culture (AVCC) of the two cell types. VECs in 3D expressed significantly higher amounts of eNOS compared to VECs in 2D. *: p < 0.05 compared to 2D. Error bars represent standard deviation.

Comparative Genomic Hybridization

To see if NanoShuttle™ affected DNA, we used comparative genomic hybridization (CGH) to observe any changes in copy number as a result of incubation with NanoShuttle™.

In doing so, we found no alterations in copy number as a result of exposure to NanoShuttle™ in monolayers of PFs. These results demonstrate that NanoShuttle™ does not lead to genomic instability.

Taken altogether, we have demonstrated through experimentation that NanoShuttle™ has no toxic or deleterious effect on cell health and function.



CGH profile of PFs in monolayers with (left) or without (right) NanoShuttle™. The bars graph the gains (right) and losses (left) 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.

References

1. Souza, G. R., J. R. Molina, R. M. Raphael, M. G. Ozawa, D. J. Stark, C. S. Levin, L. F. Bronk, J. S. Ananta, J. Mandelin, M.-M. Georgescu, J. A. Bankson, J. G. Gelovani, T. C. Killian, W. Arap, and R. Pasqualini. Three-dimensional tissue culture based on magnetic cell levitation. Nat. Nanotechnol. 5:291–6, 2010.
2. Haisler, W. L., D. M. Timm, J. A. Gage, H. Tseng, T. C. Killian, and G. R. Souza. Three-dimensional cell culturing by magnetic levitation. Nat. Protoc. 8:1940–9, 2013.
3. Timm, D. M., J. Chen, D. Sing, J. A. Gage, W. L. Haisler, S. K. Neeley, R. M. Raphael, M. Dehghani, K. P. Rosenblatt, T. C. Killian, H. Tseng, and G. R. Souza. A high-throughput three-dimensional cell migration assay for toxicity screening with mobile device-based macroscopic image analysis. Sci. Rep. 3:3000, 2013.
4. Food and Drug Administration. Generally recognized as safe (GRAS) notice (GRN). No. 135, 2004.
5. Food and Drug Admininstration. Code of federal regulations (CFR). 21 §186.1374, 2015.
6. Connor, E. E., J. Mwamuka, A. Gole, C. J. Murphy, and M. D. Wyatt. Gold nanoparticles are taken up by human cells but do not cause acute cytotoxicity. Small 1:325–7, 2005.
7. Libutti, S. K., G. F. Paciotti, A. A. Byrnes, H. R. Alexander, W. E. Gannon, M. Walker, G. D. Seidel, N. Yuldasheva, and L. Tamarkin. Phase I and pharmacokinetic studies of CYT-6091, a novel PEGylated colloidal gold-rhTNF nanomedicine. Clin. Cancer Res. 16:6139–49, 2010.
8. Lee, J., D. K. Chatterjee, M. H. Lee, and S. Krishnan. Gold nanoparticles in breast cancer treatment: promise and potential pitfalls. Cancer Lett. 347:46–53, 2014.
9. Daquinag, A. C., G. R. Souza, and M. G. Kolonin. Adipose tissue engineering in three-dimensional levitation tissue culture system based on magnetic nanoparticles. Tissue Eng. Part C. Methods 19:336–44, 2013.
10. Tseng, H., J. A. Gage, R. M. Raphael, R. H. Moore, T. C. Killian, K. J. Grande-Allen, and G. R. Souza. Assembly of a three-dimensional multitype bronchiole coculture model using magnetic levitation. Tissue Eng. Part C. Methods 19:665–75, 2013.
11. Tseng, H., L. R. Balaoing, B. Grigoryan, R. M. Raphael, T. C. Killian, G. R. Souza, and K. J. Grande-Allen. A three-dimensional co-culture model of the aortic valve using magnetic levitation. Acta Biomater. 10:173–82, 2014.
12. Tseng, H., J. A. Gage, T. Shen, W. L. Haisler, S. K. Neeley, S. Shiao, J. Chen, P. K. Desai, A. Liao, C. Hebel, R. M. Raphael, J. L. Becker, and G. R. Souza. A spheroid toxicity assay using magnetic 3D bioprinting and real-time mobile device-based imaging. Sci. Rep. 5:13987, 2015.
13. Molina, J. R., Y. Hayashi, C. Stephens, and M.-M. Georgescu. Invasive glioblastoma cells acquire stemness and increased Akt activation. Neoplasia 12:453–63, 2010.
14. Castro-Chavez, F., K. C. Vickers, J. S. Lee, C.-H. Tung, and J. D. Morrisett. Effect of lyso-phosphatidylcholine and Schnurri-3 on osteogenic transdifferentiation of vascular smooth muscle cells to calcifying vascular cells in 3D culture. Biochim. Biophys. Acta 1830:3828–34, 2013.
15. Haisler, W. L., D. M. Timm, J. A. Gage, H. Tseng, T. C. Killian, and G. R. Souza. Three-dimensional cell culturing by magnetic levitation. Nat. Protoc. 8:1940–9, 2013.

See our full list of publications on our website