Crimson Publishers
Wings to the Research

Review Article

A Leap towards 3D in Biomedicine: Do We Know How to Deal With it?

Bahaa Daou[1], Julian Zayas-Arrabal[1], Nerea Pascual[2], Alessandro Silvestri[1]* and Nuria Alegret[1,2]*

[1]Carbon Bionanotechnology Group, Center for Cooperative Research in Biomaterials (CIC Bioma GUNE), Basque Research and Technology Alliance (BRTA), Donostia-San Sebastián, 20014, Spain

[2]Group of Neuromuscular Diseases, Biodonostia Health Research Institute (HRI), Paseo Dr. Begiristain, s/n, 20014 San Sebastián, 20014, Spain

ISSN: 2637-8078

Abstract

Real life is made in three dimensions. Biological studies, however, have been for a long time based on 2D *in vitro* cultures. In the past decade, scientists have started to realize that 3D cell culturing methods are essential not only because they provide a similar environment to the *in vivo* tissue, but also because the cellular behavior in 3D better mimics the real biological scenario. Therefore, 3D scaffolds are having tremendous impact in several fields of modern biomedical research. However, even though with the hype this multidisciplinary field is having, there are multiple concerns regarding lack of handling and evaluating protocols that pose an uncertainty. Herein, we present our subjective reflection and our concerns on the matter, including the limitations and challenges we face from our experience working within such a field, aimed at leaving a mark on the consciousness of the 3D community.

Keywords: Biomedical research; Environment; 3D community; 3D cell cultures; Drug testing

***Corresponding author:** Alessandro Silvestri and Nuria Alegret, Carbon Nanobiotechnology Group, CIC bioma GUNE, Paseo de Miramón 182, 2014 Donostia-San Sebastián, and Group of Neuromuscular Diseases, IIS Biodonostia, Paseo Dr. Begiristain s/n, 20014 San Sebastian, Spain

Submission: August 25, 2021
Published: September 24, 2021

Volume 5 - Issue 3

How to cite this article: Bahaa D, Zayas Arrabal J, Nerea P, Alessandro S and Nuria A. A Leap towards 3D in Biomedicine: Do We Know How to Deal With it?. Significances of Bioengineering & Biosciences. 5(3). SBB. 000613. 2021.
DOI: 10.31031/SBB.2021.05.000613

Importance of the Third Dimension: 3D Dictates Real Geometry

In real tissue, cells are integrated in Extracellular Matrix (ECM) and interact with plenty of biochemical, mechanical, electrical stimuli, among others [1]. Current *in vitro* tests rely on bidimensional cell cultures, and do not fully embrace the complexity and heterogeneity of real biological systems. One alternative might be animal models; however, their high cost, time-consuming screenings, the need of experienced personnel, ethical issues and the fact that they do not always mimic human condition and diseases accurately, make them unfeasible for most projects. Thus, 3D cells cultures have appeared as a cost-effective ultimate answer for biomedical applications, and, with them, 3D scaffolds have gained increased attention as substrates for the tridimensional disposition of cells. Even if 3D cell cultures are not as widespread or standardized as 2D cell culture, its publications have grown exponentially since 1995 [1,2]. Historically, they were first used for drug testing in cancer biology due to their capacity to mimic *in vivo* aspects of the phenotype and cellular heterogeneity, *e.g.,* the microenvironment of the tumor growth; nowadays, the variety of manufacturing methodologies allows to accurately reproduce *in vivo* tissues, thus their range of applications has been extended widely [3,4]. Several studies demonstrated that 3D scaffolds or substrates with rich and interconnected pores enhance cell shape, exposure to medium, differentiation and proliferation, and that such cells have a behaviour and response that is closer to the *in vivo*, providing a more realistic predictive outcome [2,5,6]. Morphologically, 3D scaffolds provide a better adhesion onto the surface and the multi-cellular interactions are known for promoting prolonged cell survival [6,7]. Moreover, 3D scaffolds allow the formation of multicellular aggregates, or spheroids, which creates tridimensional interactions with cells and the ECM [8]. Functionally, the markers and protein expression are remarkably better than in monolayer cultures and the surface area increases, making 360° interaction with neighbouring cells possible, which promotes cell or biomaterial attachment, proliferation, sensing, etc., [7,9].

Several lines of evidence showed that 3D cultures recapitulate *in vivo* cell-to-cell and cell-to-matrix interactions and express unique and desirable behaviour, response and cellular characteristics and supporting their use in basic and translational research [5,10].

What to consider 3D vs 2D scaffolds

The tridimensional feature has been referred in multiple ways, given the recent novelty of this concept in biomedicine; but there is not always a clear distinction between 2 and 3 dimensionality. In literature 3D has been assigned to structures with thicknesses either of few millimeters, few microns and even few nanometers, as fibers or membranes [11-13]. Furthermore, pore size becomes fundamental when dealing with 3D cultures. For instance, traditional fibrous meshes, even those of mm thickness, can have pore sizes up to few microns, thus providing superficial porosity but hindering cell infiltration and hampering achieving a 3D culture [14,15]. Thus, we consider necessary for the 3D scaffold's community to define the limits of tridimensionality. Our own suggestion would be the length in each direction must be a minimum of double the average height of the studied cell. For example, if cardiomyocytes are incubated, with known thickness of around 20μm, [16] the 3D scaffold must have a height of at least 40μm. Revisiting the literature, not a large percentage of the publications specify a conductive 3D scaffold under our definition. We believe that these characteristics (dimensions of the 3D and pore size) are essential to obtain reliable predictive results in biomedical applications.

Significance of 3D Cultures in Biomedical Research

3D cell culturing methods have the potential to solve the locked questions unable to answer using 2D cell culture techniques. For this reason, they are having tremendous impact in several fields of modern biomedical research. The development of 3D cell cultures is being central to elucidate biological, mechanical and chemical signals that are needed for the cells to proliferate and differentiate, secrete extracellular matrix and form functional tissues. For instance, a recent study has shown that the 3D culturing of human adipose-deriving stem cells enhances their pluripotency and differentiation abilities [17]. Another recent publication has reported the mechanical and molecular parameters that influence tendon differentiation [18]. Then, besides providing a better knowledge about proliferation and differentiation, 3D cultures systems that can regulate proliferation and differentiation of cells like pluripotential stem cells are key in the development of regenerative medicine [19]. Tissue engineering and regenerative medicine are among the most explored application of 3D cultures: when designing an artificial cellular model, it is imperative that the micro-environment accurately mimics the target human tissue [20]. With this aim, numbers of papers have been recently published employing Extracellular Matrixes (ECMs) to create artificial 3D scaffolds and hydrogels with properties analogues to primary tissues [20-22]. Furthermore, the incorporation of nanomaterials and conductive polymers in 3D scaffolds is an open field that allows to integrate functions cell culture with cellular differentiation, electrical conductivity, mechanical stimulation, and real-time detection capability [23-25].

In addition, 3D cell culture systems, are becoming prominent as well in drug discovery and repositioning. Their ability to model *in vivo* microenvironments ensures high predictive value for clinical outcome and, at the same time, circumvents the mouse models drawbacks. Highly innovative works have been recently published in this field, boosting the development of efficient, rapid, and personalized platform for drug screening [26,27]. Both optical/fluorescent microscopy and electrochemical impedance has been explored to monitor cell proliferation/apoptosis [26,28]. 3D culture has been combined with microfluidics and combinatorial approaches to automated and high-throughput platform that facilitates preclinical research and personalized therapies [28]. Moreover, personalized drug screening has been achieved using patient-based organoids, demonstrating significant differences in the drug response from patient to patient [26].

On the other hand, 3D cell culture also represents a great opportunity to better understand cellular and molecular mechanisms, more specifically the key role of the tumor microenvironment, involved in the development and establishment of solid tumors. In this sense, 3D cell culture, but not 2D culture, can better recreate a sort of tissue microenvironment, providing more accurate data about tumorigenesis such as cell to-cell interactions or cell-to-extracellular matrix interactions [2]. With 3D cell cultures, it is possible to co-cultivate epithelial and stromal cells and observe the crosstalk of multiple cell types interacting, which regulate normal and neoplastic development [29], as well as to create aggregates of cells, producing several common features that are similar to the solid tumor *in vivo,* such as cellular heterogeneity, cell-cell signaling, hypoxia, membrane protein distribution, and gene expression patterns [30]. In particular, 3D cell culture are helping in untangling the mechanisms at the base of cancer cellular signaling. Various other studies have observed that 3D cultures show distinct differences from their 2D counterparts in terms of cell signaling pathways [31]. For example, it has been reported that cancer cells cultivated in 3D ECM presents reduced phosphatide inositol 3 kinase pathway compared to 2D cultures, which is responsible of the aggressive cell growth and invasion in tumors [32]. Analogously, it was observed that, for cervical cancer cells, 2D derived extracellular vesicles showed different profiles in terms of secretion dynamics and signaling molecules contents compared to the 3D- culture derived [33].

Handling 3D samples: Current Challenges and Limitations

Up to date, a large variety of biocompatible polymers, dopants, compositions and biomaterials have been used in the design 3D

scaffolds [9]. The main drawbacks in 3D cultures is the difficulty of construction and replication, higher costs and large amount of effort needed. In that line, 3D printing is arising as a promising technique to automate and increase the bulk production of 3D scaffold fabrication [34]. In contrast to conventional techniques, 3D printing allows the fabrication of customized scaffolds with controlled shape, pore size and pore structure through a precise layer-by-layer deposition [35]. Many of the 3D scaffolds have great potential to mimic features of larger tissues or entire organs, although there is still a huge gap between material engineering and biomedical application to be solved. In this reflection, we think it is important to highlight the lack of methodological manners, which are less well-known, and the urgency in defining proper preparation, characterization and evaluation protocols. This will not only reduce the gap and homogenize the communication between scientists, but also allow to rapidly move forward to the final biomedical goal.

Questioning the efficacy of common sterilization techniques of 3D biomaterials

When it comes to 3D biomaterials, it has become evident that good sterilization techniques before cell studies and pre-clinical trials is crucial. The obvious choice is going to conventional sterilization methods, which include thermal stabilization (Dry heat, Steam heat), Ethylene oxide sterilization, or chemical sterilization [36,37]. However, as 3D biomaterials are designed to fit the biocompatibility profile of specific biological tissues, their specific chemical and physical properties are often prone to rapid exacerbation during these processes. For instance, thermally sensitive polymers that have their glass transition temperature (T_g) lower than the sterilization temperature lose their shape and can undergo phase inversion; others are reactive to solvents used in chemical sterilization. UV irradiation is considered among the safest, quickest sterilization process with medium inactivation strength [38]. In a recent study, Tapia-Guerrero [38] demonstrated that sterilization by UV and Gamma irradiation of Poly (ε-caprolactone) (PCL) and Poly (Lactic-Co-Glycolic Acid) (PLGA) polymeric nanoparticles proved safe and preserved the chemical integrity of those nanoparticles [39].

Nonetheless, Gamma irradiation even at relatively low doses (5 and 10kGy) were slightly modifying the mean particle size and zeta potential. On the other side, 3D biomaterials are engineered to protect cells, increase resistance to cell degradation and provide a physical medium for cellular proliferation. These properties often signify that also other microorganisms will favor their own proliferation in such materials. Moreover, they would prove even more resistant inside these scaffolds given that irradiation with safe doses of UV light is already less effective towards certain microorganisms on 2D surfaces such as mycobacteria, bacteria spores, and Prions [40]. To avoid biological contamination, scientists often use other harsher sterilization methods without considering possible change in the physical and chemical structures of 3D biomaterials.

Another sterilization method proved to be safe and effective is based on supercritical CO_2 ($scCO_2$). Santos-Rozales [40] studied the $scCO_2$ sterilization of PCL/PLGA scaffolds in the presence of H_2O_2 as a co-solvent and demonstrated efficient microbial inactivation while keeping the structure and chemical properties of the hydrogels [41]. Bernhardt [41] studied the effects of $scCO_2$ sterilization in alginate and collagen based hydrogels at low temperature in the presence of H_2O_2 and acetic anhydride as co-solvents reporting similar results of structural and chemical preservation [42]. However, in both articles listed above the authors reported an increase in the compressive modulus after $scCO_2$ treatment, which in the first place is tweaked to mimic the exact biological nature of the tissue under study.

Viability analyses: false positive or inefficient sterilization?

Another critical point to highlight is related to the cytotoxicity studies. The vast majority are based on colorimetric assays of the detection of a certain indicator molecule produced by the cells upon their death, e. g., the quantification of Lactate Dehydrogenase (LDH). Such methodologies can be hampered from the fact that the 3D biomaterials can enclose or adsorb the reporting molecules within their structure. This means that the optical density of control samples (usually 2D) may show higher value than those containing cells seeded onto 3D biomaterials. Indeed, there is a need to define new kind of 3D blanks samples or even new cytotoxicity/viability protocols that would give a real and high viability comparable to those 2D cultures.

Cell visualization inside a 3D matrix

Imaging becomes difficult in large scales: fluorescence does not allow z-stack images and, therefore, cells grown in a biomimetic 3D geometry are commonly visualized under confocal microscopy [35], which allows 3D imaging but is not fast enough to map a significative portion of the sample. In fact, 3D in confocal imaging applies for few hundreds of micrometers in thickness in the most favorable cases, but certainly does not allow a full visualization of a millimeter-size culture. Furthermore, the resolution of the resulting 3D images is quite low, and the characteristic features of the cell phenotype are usually lost, e.g., the stripped sarcomere of cardiomyocytes becomes blurry [43]. In our own experience, the best accurate images of cellular distribution and phenotype can be obtained from conventional 2D imaging microscopy techniques, as SEM microscopy. Nokoorani [43] showed an example of FE-SEM imaging of cells incubated in chitosan/gelatin-based scaffolds after fixation with glutaraldehyde at different time intervals. After multiple efforts and tests performed in our laboratory, we argue that the current imaging techniques do not fully reflect the reality of the cell-to-cell nor the cell-to-matrix interaction within the 3D

matrix in the tridimensional geometry. Usually, the approach is based on slicing the scaffold in thin layers, imaging them separately and finally reconstructing a 3D image [43]. Even so, this invasive method alters the cell morphology, interactions, and cell-material interfaces, yielding to a loss of some information, especially in cut-off areas of the texture. Furthermore, a lot of manipulating problems appear, such as folding, pulling, and tearing. This field is increasing in the past years, and the trend seems to combine tissue clearing with new microscopy methods, such as Light Sheet Fluorescence Microscope (LSFM), and image analysis software. However, this is not yet a routine.

Conclusion

3D substrates have emerged as the next generation platforms to translate the in-laboratory experiments to the real *in vivo*, thus providing a cost-effective reliable tool to predict cellular behaviors and responses before subsequent clinical trials. Nevertheless, this field is in its early stages, and we anticipate that it will be exponentially expanded in the coming years, and new methodologies to achieve the 3D architecture will be developed to meet the demands of clinical trials. The main areas were 3D environments have the most potential are in tissue engineering, of tissue, drug delivery. The current studies are focused on *in vitro* assays that, although they are suitable for an initial prediction, await the translation to real cases. The usual concerns are addressed in the design of the scaffold (structure, composition, properties) to fit all the necessities for the desired application. However, our concern relies of the manipulation and analyses of the 3D systems used, as it is not a common practice to define protocols, e.g., sterilization and preparation for imaging, in the published articles. It is our desire that this reflection will enlighten the awareness that the elemental limitations must be solved to achieve a better praxis and transparency, good understanding and reducing the gap between the materials engineers and biomedical community.

References

1. Zuppinger C (2019) 3D cardiac cell culture: A critical review of current technologies and applications. Frontiers in Cardiovascular Medicine 6: 87.
2. Jensen C, Teng Y (2020) Is it time to start transitioning from 2d to 3d cell culture? Frontiers in Molecular Biosciences 7: 33.
3. Souza AG, Silva IBB, Campos-Fernandez E, Barcelos LS, Souza JB, et al. (2018) Comparative assay of 2d and 3d cell culture models: Proliferation, gene expression and anticancer drug response. Curr Pharm Des 24(15): 1689-1694.
4. Imamura Y, Mukohara T, Shimono Y, Funakoshi Y, Chayahara N, et al. (2015) Comparison of 2D- and 3D-culture models as drug-testing platforms in breast cancer. Oncol Rep 33(4): 1837-1843.
5. Maddaly R, Kaviya SR, Paramesh V, Anuradha E, Solomon FDP (2015) 3D cell culture systems: advantages and applications. J Cell Physiol 230(1): 16-26.
6. Langhans SA (2018) Three-dimensional *in vitro* cell culture models in drug discovery and drug repositioning. Frontiers in Pharmacology 9: 6.
7. Jamalpoor Z, Soleimani M, Taromi N, Asgari A (2019) Comparative evaluation of morphology and osteogenic behavior of human Wharton's jelly mesenchymal stem cells on 2D culture plate and 3D biomimetic scaffold. J Cell Physiol 234(12): 23123-23134.
8. McKee C, Chaudhry GR (2017) Advances and challenges in stem cell culture. Colloids Surf B Biointerfaces 159: 62-77.
9. Choi JS, Park JS, Kim B, Lee BT, Yim JH (2017) *In vitro* biocompatibility of vapour phase polymerised conductive scaffolds for cell lines. Polymer 124: 95-100.
10. Ylostalo JH (2020) 3D stem cell culture. Cells 9(10): 2178.
11. Lee H, Cho Y (2015) An innovative strategy for the fabrication of functional cell sheets using an electroactive conducting polymer. Theranostics 5(9): 1021-1029.
12. Mihailescu M, Popescu RC, Matei A, Acasandrei A, Paun IA (2014) Investigation of osteoblast cells behavior in polymeric 3D micropatterned scaffolds using digital holographic microscopy. Appl Opt 53(22): 4850-4858.
13. Gelmi A, Zhang J, Cieslar-Pobuda A, Ljunngren MK, Los MJ, et al. (2015) Electroactive 3D materials for cardiac tissue engineering. In Electroactive Polymer Actuators and Devices (EAPAD).
14. Lee JY, Bashur CA, Goldstein AS, Schmidt CE (2009) Polypyrrole-coated electrospun PLGA nanofibers for neural tissue applications. Biomaterials 30(26): 4325-4335.
15. Jin L, Wang T, Feng ZQ, Zhu M, Leach MK, et al. (2012) Fabrication and characterization of a novel fluffy polypyrrole fibrous scaffold designed for 3D cell culture. J Mater Chem 22(35): 18321-18326.
16. Tracy RE (2012) Cardiomyocyte size estimated from noninvasive measurements of left ventricular wall thickness and chamber diameter. J Am Soc Hypertens 6(3): 185-192.
17. Sung TC, Heish CW, Lee HHC, Hsu JY, Wang CK, et al. (2021) 3D culturing of human adipose-derived stem cells enhances their pluripotency and differentiation abilities. Journal of Materials Science & Technology 63: 9-17.
18. Gaut L, Bonnin MA, Blavet C, Cacciapuoti I, Orpel M, et al. (2020) Mechanical and molecular parameters that influence the tendon differentiation potential of $C_3H_{10}T_{1/2}$ cells in 2D- and 3D-culture systems. Biol Open 9(2).
19. Boland MJ, Hazen JL, Nazor KL, Rodriguez AR, Gifford W, et al. (2009) Adult mice generated from induced pluripotent stem cells. Nature 461(7260): 91-94.
20. Hussey GS, Dziki JL, Badylak SF (2018) Extracellular matrix-based materials for regenerative medicine. Nature Reviews Materials 3(7): 159-173.
21. Zhu M, Li W, Dong X, Yuan X, Midgley AC, et al. (2019) In vivo engineered extracellular matrix scaffolds with instructive niches for oriented tissue regeneration. Nature Communications 10(1): 4620.
22. Chiang CE, Fang YQ, Ho CT, Assunção M, Lin SJ, et al. (2021) Bioactive decellularized extracellular matrix derived from 3D stem cell spheroids under macromolecular crowding serves as a scaffold for tissue engineering. Advanced Healthcare Materials 10(11).
23. Qin Y, Hu XB, Fan WT, Yan J, Cheng SB, et al. (2021) A Stretchable Scaffold with Electrochemical Sensing for 3D Culture, Mechanical Loading, and Real-Time Monitoring of Cells. Advanced Science 8(13): 2003738.
24. Dominguez AA, Alegret N, Arnaiz B, Salsamendi M, Mecerreyes D, et al. (2020) Toward spontaneous neuronal differentiation of SH-SY5Y cells using novel three-dimensional electropolymerized conductive scaffolds. ACS Applied Materials & Interfaces 12(51): 57330-57342.
25. Alegret N, Dominguez AA, González DJM, Arnaiz B, Cossío U, et al. (2018) Three-dimensional conductive scaffolds as neural prostheses based on carbon nanotubes and polypyrrole. ACS Applied Materials & Interfaces 10(50): 43904-43914.

26. Schuster B, Junkin M, Kashaf SS, Romero CI, Kirby K, et al. (2020) Automated microfluidic platform for dynamic and combinatorial drug screening of tumor organoids. Nature Communications 11(1): 5271.

27. Yan X, Zhou L, Wu Z, Wang X, Chen X, et al. (2019) High throughput scaffold-based 3D micro-tumor array for efficient drug screening and chemosensitivity testing. Biomaterials 198: 167-179.

28. Pan Y, Hu N, Wei X, Gong L, Zhang B, et al. (2019) 3D cell-based biosensor for cell viability and drug assessment by 3D electric cell/matrigel-substrate impedance sensing. Biosensors and Bioelectronics 130: 344-351.

29. Sawant S, Dongre H, Singh AK, Joshi S, Costea DE, et al. (2016) Establishment of 3D Co-culture models from different stages of human tongue tumorigenesis: Utility in understanding neoplastic progression. PLOS ONE 11(8): e0160615.

30. Zhao L, Shi M, Liu Y, Zheng X, Xiu J, et al. (2019) Systematic analysis of different cell spheroids with a microfluidic device using scanning electrochemical microscopy and gene expression profiling. Analytical Chemistry 91(7): 4307-4311.

31. Ravi M, Paramesh V, Kaviya SR, Anuradha E, Solomon FDP (2015) 3D Cell culture systems: Advantages and applications. Journal of Cellular Physiology 230(1): 16-26.

32. Fallica B, Maffei JS, Villa S, Makin G, Zaman M (2012) Alteration of cellular behavior and response to PI3K pathway inhibition by culture in 3D collagen gels. PLOS ONE 7(10): e48024.

33. Thippabhotla S, Zhong C, He M (2019) 3D cell culture stimulates the secretion of *in vivo* like extracellular vesicles. Sci Rep 9(1): 13012.

34. Ligon SC, Liska R, Stampfl J, Gurr M, Mülhaupt R (2017) Polymers for 3D printing and customized additive manufacturing. Chem Rev 117(15): 10212-10290.

35. Cestari F, Petretta M, Yang Y, Motta A, Grigolo B, et al. (2021) 3D printing of PCL/nano-hydroxyapatite scaffolds derived from biogenic sources for bone tissue engineering. Sustainable Materials and Technologies 29: e00318.

36. Phillip Jr E, Murthy NS, Bolikal D, Narayanan P, Kohn J, et al. (2013) Ethylene oxide's role as a reactive agent during sterilization: Effects of polymer composition and device architecture. Journal of Biomedical Materials Research Part B: Applied Biomaterials 101B(4): 532-540.

37. Evrova O, Kellenberger D, Scalera C, Calcagni M, Giovanoli P, et al. (2019) Impact of UV sterilization and short term storage on the *in vitro* release kinetics and bioactivity of biomolecules from electrospun scaffolds. Scientific Reports 9(1): 15117.

38. Tapia Guerrero YS, Del Prado Audelo ML, Borbolla Jiménez FV, Giraldo Gomez, DM, García Aguirre I, et al. (2020) Effect of UV and gamma irradiation sterilization processes in the properties of different polymeric nanoparticles for biomedical applications. Materials 13(5): 1090.

39. McDonnell G, John Wiley & Sons, UK, pp. 149-247.

40. Santos RV, Magariños B, Starbird R, Suárez GJ, Fariña JB, et al. (2021) Supercritical CO_2 technology for one-pot foaming and sterilization of polymeric scaffolds for bone regeneration. International Journal of Pharmaceutics 605: 120801.

41. Bernhardt A, Wehrl M, Paul B, Hochmuth T, Schumacher M, et al. (2015) Improved sterilization of sensitive biomaterials with supercritical carbon dioxide at low temperature. PLOS ONE 10(6): e0129205.

42. Nowzari F, Wang H, Khoradmehr A, Baghban M, Baghban N, et al. (2021) Three-dimensional imaging in stem cell-based research. Frontiers in Veterinary Science 8(352): 657525.

43. Nokoorani YD, Shamloo A, Bahadoran M, Moravvej H (2021) Fabrication and characterization of scaffolds containing different amounts of allantoin for skin tissue engineering. Scientific Reports 11(1): 16164.